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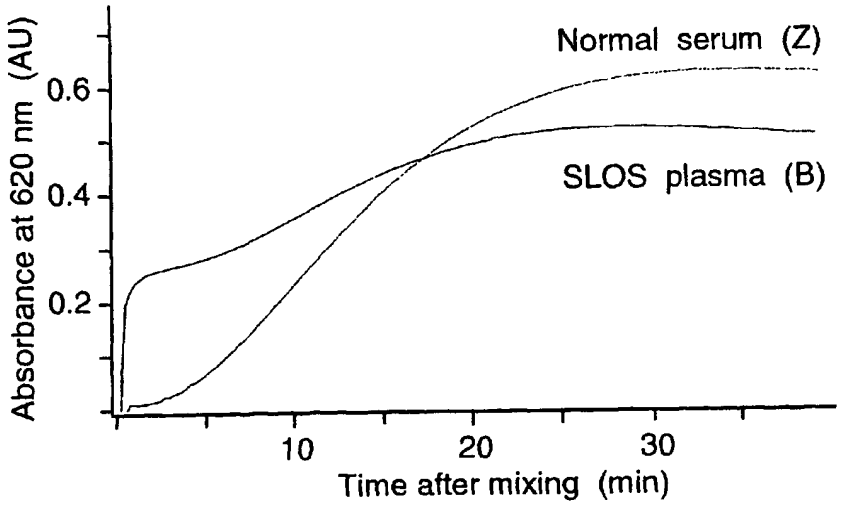
(54) Title: PRACTICAL SCREENING METHOD FOR DETECTING GENETIC DISORDERS OF STEROL METABOLISM



(57) Abstract: An assay is described to screen for genetic disorders of sterol metabolism based on differences in a visual and qualitative colorimetric response and a quantitative spectrophotometric response to the Liebermann-Burchard reagent. The invention describes a qualitative and quantitative colorimetric screen for Smith-Lemli-Opitz syndrome (SLOS). The invention also relates to diagnosis of SLOS by nuclear magnetic resonance of a sample containing sterols, which can distinguish mild cases of SLOS from normal subjects by determination of individual noncholesterol sterols. Further, the invention describes the treatment of Guthrie cards to delay, slow, or prevent decomposition of sterols during storage.

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PRACTICAL SCREENING METHOD FOR DETECTING GENETIC DISORDERS OF STEROL METABOLISM

[0001] The work herein was supported by grants from the United States Government. The United States Government may have certain rights in the invention.

FIELD OF THE INVENTION

[0002] The invention generally relates to the fields of biochemistry, chemistry and medicine and to a method for detecting genetic disorders of sterol metabolism using visual and spectroscopic response of a sample of blood or other body fluid to Liebermann-Burchard reagent to distinguish a diseased subject from a normal human subject. The present invention particularly relates to a method for detecting Smith-Lemli-Opitz Syndrome (SLOS), a hereditary developmental disorder characterized by aberrant sterol accumulation.

BACKGROUND OF THE INVENTION

[0003] The preferred sterol in animal systems is cholesterol. Cholesterol is an essential membrane sterol that aids in maintaining homeostatic membrane fluidity and permeability, serves as a synthetic precursor for bile acids, steroid hormones, and some vitamins. Cholesterol is also very important for fetal development and normal growth during childhood. the population at large is continually advised that it is prudent to know serum cholesterol levels and constantly reminded that an uncontrolled diet and a lack of exercise can lead to accumulation of arterial plaque that increase the risk of atherosclerosis and coronary heart disease. Because of the clinical importance of cholesterol, numerous studies have been described that quantitate serum cholesterol levels. Cholesterol analysis in skin using the Liebermann-Burchard reagent has been described by Moore *et al.*, 1952. In this experiment, cholesterol was quantified by determining the optical density of rat skins, following saponification and isolation of the nonsaponifiable lipids, by measuring the intensity of a resulting color observed between 30 and 35 minutes after addition of the Liebermann-Burchard reagent. The Liebermann-Burchard reagent has proven useful in estimating serum sterol levels.

[0004] Cholesterol is the major sterol biosynthesized in humans. Disorders in sterol metabolism lead to accumulation of aberrant sterols and sterol intermediates and result in severe dysmorphogenic syndromes of variable severity. These metabolic disorders of sterol metabolism result in elevated levels of sterol biosynthetic intermediates and include Smith-Lemli-Opitz syndrome (SLOS), Conradi-Hunermann syndrome, desmosterolosis, and Greenberg dysplasia. SLOS is among several known genetic disorder of sterol metabolism in which the anabolism *de novo* of cholesterol is prematurely terminated. SLOS is an autosomal recessive syndrome characterized by microencephaly, growth retardation, mid-face dysplasia, syndactyly, polydactyly, cataracts, heart and kidney malformations, and mental retardation. The frequency of genetic mutations that cause SLOS has been estimated at roughly 1 in 100, based on observed incidences of roughly between 1/20,000 and 1/60,000 live births (Ryan *et al.*, 1998; Lowry *et al.*, 1980). The carrier frequency for SLOS was recently described as about 1 in 30, corresponding to an incidence of between 1/1590 and 1/13,500 conceptions (Battaile et al., 2001). In any case, SLOS is one of the most common recessively inherited disorders among populations of European heritage. Phenotypic variation makes the diagnosis difficult, and it is clear that SLOS is underdiagnosed.

[0005] Disorders in sterol metabolism result in dysmorphogenic syndromes of variable severity. Conradi-Hunermann syndrome represents a genetic disorder of sterol metabolism characterized by the accumulation of cholesta-8(9)-en-3β-ol and 8-dehydrocholesterol resulting from function-impairing mutations in sterol-Δ^8-isomerase (DiPreta *et al.*, 2000). This disease is an X-linked dominantly inherited chondrodysplasia punctata form that is evidenced by malformation of the extremities, cataracts, cutaneous lesions, and an unusual facies. Desmosterolosis is caused by a defect of desmosterol reductase (3β-hydroxysteroid-Δ^{24}-reductase) causing an accumulation of cholest-5,24-dien-3β-ol and induces a severe osteosclerotic skeletal dysplasia with multiple embryonic malformations similar to those observed in SLOS. CHILD syndrome, congenital hemidysplasia with ichthyosis and limb defects, is a rare genetic disorder that has been linked to mutations in sterol-Δ^8-isomerase and sterol-4-demethylase. Like Conradi-Hunermann syndrome, cutaneous and bony abnormalities are pathologies observed in CHILD syndrome. Greenberg dysplasia, also known as HEM, is an autosomal recessive disease that often causes lethal skeletal dysplasia. Greenberg dysplasia has been linked to

mutations in sterol-D14-isomerase, thereby accumulating 14-dehydrocholesterols including cholesta-8,14-dien-3β-ol and cholesta-8,14,24-trien-3β-ol.

[0006] Medical research indicates that treatment of such disorders and prenatal or early postnatal intervention may greatly improve the outcomes of the sufferers. Neonatal screening has become standard practice in most industrialized nations with virtually every newborn being routinely screened for phenylketonuria (PKU) and congenital hypothyroidism and this early screening has been shown to be cost-effective (Pediatrics, 2000). Both conditions result in severe mental retardation if not diagnosed in the immediate newborn period and started on treatment. Many newborn screening programs have also added tests for additional conditions including sickle cell anemia, galactosemia, maple syrup urine disease, homocystinuria, congenital adrenal hyperplasia, biotinidase deficiency, and cystic fibrosis.

[0007] SLOS results from an inborn error in cholesterol metabolism. The specific defect is caused by mutations in the gene for 7-dehydrocholesterol Δ7-reductase (DHCR7). This enzyme converts 7-dehydrocholesterol (7DHC) to cholesterol, the final step of cholesterol biosynthesis (**1**). The genetic mutations that have been characterized in SLOS patients include at least 73 different 7-dehydrocholesterol 7-reductase mutations, which consist of 65 missense mutations and several null mutations, including two nonsense mutations, one splice site mutation, two single nucleotide insertions, and three deletions (Witsch-Baumgartner et al., 2001; Yu *et al.*, 2000). Because of the numerous genetic possibilities, screening DNA has not provided a reliable diagnostic tool for SLOS. In fact, the highest occurrence frequency of a common genetic mutation has been described to be a point mutation that occurs in about 60% of the U.S. propositi (Yu *et al.*, 2000). SLOS subjects are homozygous for defective DHCR7, and the absence of both copies of a normal DHCR7 gene results in a deficiency of cholesterol and an accumulation of 7DHC and related noncholesterol sterols in blood and tissues.

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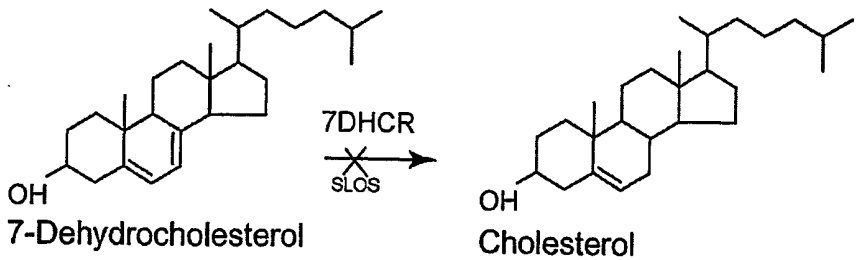

7-Dehydrocholesterol Cholesterol

[0008] The major noncholesterol sterols in SLOS are the $\Delta^{5,7}$ and $\Delta^{5,8}$ dienes, which have been found in blood, other fluids, tissues, and feces. Many additional sterols have been reported to be present in SLOS (Tint *et al.*, 1995; Salen *et al.*, 1996; Batta *et al.*, 1994; Kelley, 1994, Kelley, 1995; Rossiter *et al.*, 1996) or animal models (Wolf *et al.*, 1996; Llirbat *et al.*, 1997): 19-norcholesta-5,7,9-trien-3β-ol (Salen *et al.*, 1996; Batta *et al.*, 1995; De Fabiani *et al.*, 1996; Ness *et al.*, 1997; Wolf *et al.*, 1996; Llirbat *et al.*, 1997), 24,25-dihydrolanosterol (Kelley, 1994), and $\Delta^{5,7,9(11)}$ (De Fabiani *et al.*, 1996), Δ^{7} (8, Kelley, 1994; Kelley, 1995; Llirbat *et al.*, 1997), Δ^{8} (Llirbat *et al.*, 1997), $\Delta^{5,24}$ (Tint *et al.*, 1995), $\Delta^{6,8}$ (1, Kelley, 1995;Rossiter *et al.*, 1995), $\Delta^{6,8(14)}$ (Batta *et al.*, 1994), $\Delta^{8(14)}$ (Kelley, 1995), and $\Delta^{5,7,24}$ (Batta *et al.*, 1994; Kelley, 1995) sterols. Most of these substances were characterized as minor components of complex sterol mixtures by gas chromatography-mass spectrometry (GC-MS) or GC although some analyses were done by high performance liquid chromatography (HPLC) (Ness *et al.*, 1997), HPLC-MS (De Fabiani *et al.*, 1996), or thin-layer chromatography (TLC) followed by GC-MS (Tint *et al.*, 1995; Batta *et al.*, 1995) and other characterization (Batta *et al.*, 1995). Except for fecal sterols (Batta *et al.*, 1995), identifications were generally based on comigration with an authentic standard on a single chromatographic phase, sometimes in conjunction with mass spectrometry. Although facile, these chromatographic and spectral methods alone are poorly suited to the comprehensive analysis of complex sterol mixtures. Detailed investigations of the GC, GC-MS, and HPLC of sterols indicate serious limitations in their use for the separation and identification of unsaturated C_{27} sterols (Gerst *et al.*, 1997; Ruan *et al.*, 1997; Ruan *et al.*, 1996). Data from a large collection of authentic standards have shown that many pairs of sterols, including acetate and trimethylsilyl (TMS) derivatives, coelute on a variety of stationary phases commonly used in GC (Gerst *et al.*, 1997) and HPLC (Ruan *et al.*, 1997). Moreover, a number of sterol isomers relevant to SLOS cannot be distinguished by mass spectrometry under standard electron-impact conditions (Gerst *et al.*, 1997). Because sterol mixtures of any complexity are likely to contain comigrating components on GC, HPLC, or TLC, such analyses, even in conjunction with mass spectrometry, can provide only tentative identification and quantitation of the sterols present. A further complication is the lability of several sterols to GC conditions (Gerst *et al.*, 1997). For example, the $\Delta^{5,8}$ sterol partially decomposes

under the thermal stress of GC injection to form the 19-nor-$\Delta^{5,7,9}$ sterol as an artifact (Ruan *et al.*, 1996).

[0009] The unique association of SLOS with elevated 7DHC levels leads to a dramatic difference in sterol composition between normal and SLOS subjects and provides the basis for several screening methods to distinguish SLOS from normal samples. Early developmental abnormalities that are primarily responsible for the physical signs of SLOS are attributed to aberrant embryonic patterning, which involves covalent binding of cholesterol to the morphogen sonic hedgehog with concomitant autoproteolysis, followed by signal transduction by incompletely defined mechanisms (Kelley *et al.*, 2000; Porter, 2000). The severity of SLOS is related to genotype, cholesterol levels, and levels of noncholesterol sterols, but correlations with severity are confounded by additional factors (Kelley, *et al.*, 2000; Yu *et al.*, 2000). The abnormal sterol profile of the SLOS neonate evolves during childhood to produce higher but still usually depressed cholesterol levels, a process that is facilitated by cholesterol feeding (Kelley *et al.*, 2000; Porter, 2000).

[0010] Prior to the present invention, methods described in the scientific literature include analysis of the trimethylsilyl ether derivatives of the nonsaponifiable lipids by gas chromatography-mass spectrometry (GC-MS) (Kelley, 1995; Abuelo *et al.*, 1994; Kelley, 1994), ultraviolet spectral analysis of plasma extracted with hexane (Seedorf et al., 1994; Honda *et al.*, 1997), HPLC analysis (Lund *et al.*, 1996), and time-of-flight secondary-ion mass spectrometry (TOF-SIMS) (Seedorf, *et al.*, 1995; Zimmerman et al., 1997). Other SLOS screening methods are based on cell culture of fibroblasts (Honda *et al.*, 1997) GC-MS of urinary steroids (Shackelton *et al.*, 1999a; Shackelton *et al.*, 1999b; Shackelton et al., 2001), or detection of specific DHCR7 mutations by the polymerase chain reaction (Yu et al., 2001). However, most of these methods either suffer from low sensitivity, require elaborate instrumentation not normally available in clinical settings, require sample derivatization prior to analysis, or require a combination thereof.

[0011] U.S. Patent 5,629,210 describes analysis of organic samples by TOF-SIMS to screen for SLOS to determine the ratio of cholesterol/7DHC, which allows potential identification of SLOS. GC-MS of processed urine samples has allowed characterization of a patient's steroid profile, in particular the presence of 7DHC (Shackleton *et al.*, 1999a; Shackleton *et al.*, 1999b; Shackelton et al., 2001).

[0012] Honda *et al.*, 1997 describe screening for SLOS by determination of 7-dehydrocholesterol by ultraviolet spectroscopy. Plasma and cultured skin fibroblasts from SLOS patients are analyzed based on characteristic absorption maxima for 7-dehydrocholesterol at wavelengths (λ) 271, 282, and 294 nm. In SLOS patients, cholesterol levels are lowered as a result of the accumulation of 7-dehydrocholesterol and 8-dehydrocholesterol. However, cases exist in which the cholesterol level is normal and 7-dehydrocholesterol levels are lower than reported for SLOS patients (Kelley *et al.*, 1995). In order to establish a screen suitable for an atypical case of SLOS, Honda *et al.*, 1997 cultured fibroblasts in the presence of 10% fetal bovine serum which caused an increase in the 7-dehydrocholesterol. However, because existing methods for diagnosis are too expensive for screening of large populations, the prevalence of SLOS is still not clearly defined. If a therapy to treat SLOS, such as cholesterol feeding, is to be effective, an early diagnosis through newborn screening of all cases, severe to mild, is essential.

SUMMARY OF THE INVENTION

[0013] In one embodiment within the scope of the present invention, a mammal is screened for a genetic disorder of sterol metabolism comprising the steps of collecting a test sample from the mammal to be tested, extracting sterols from the test sample, concentrating a sterol extract, forming a reaction mixture by mixing the concentrated sterol extract or a solution of the concentrated sterol extract with an acid reagent, and comparing the transient color of the reaction mixture with a transient color of a control mixture to determine the presence of a genetic disorder of sterol metabolism.

[0014] In specific embodiments, the test sample is selected from the group consisting of a dried blood spot and a body fluid. The dried blood spot is generally on a Guthrie card, and preferably on a Guthrie card that has been treated with a chemical stabilizer.

[0015] In preferred specific embodiments, the chemical stabilizer retards, slows, delays, or prevents decomposition of 7-dehydrocholesterol and 8-dehydrocholesterol. A chemical stabilizer is selected from a group consisting of butylated hydroxytoluene (BHT), alpha-tocopherol (vitamin E), N-acetyl cysteine, tert-butyl phenyl nitrone, ascorbic acid (vitamin C), mannitol, and beta-carotene.

[0016] In specific embodiments, the genetic disorder is selected from the group consisting of Smith-Lemli-Opitz syndrome, Conradi-Hunermann syndrome, CHILD syndrome, desmosterolosis, and Greenberg displasis.

[0017] In another specific embodiment, the extracting of sterol from a test sample comprises the steps of adding to the test sample about 16 volumes of methanol, about 30 to 50 volumes of chloroform, and stirring for about 30 minutes or mixing on a vortex apparatus for about 1 minute.

[0018] In specific embodiments, the sterol extract is concentrated by removing liquid from the sterol extract.

[0019] In another specific embodiment, the acid reagent is Liebermann-Burchard reagent. In a preferred specific embodiment, the Liebermann-Burchard reagent is modified by diluting with chloroform.

[0020] In further specific embodiments, the concentrated sterol extract or solution of the concentrated sterol extract is at a first subambient temperature and the acid reagent, which is at a second subambient temperature are mixed. Also within the scope of the present invention, the mixing comprises a stopped-flow cell apparatus, and preferably the stopped-flow cell apparatus is at a subambient temperature. In another specific embodiment, a positive test is determined by visualization of a transient color at about 5 seconds following the addition of the acid reagent. A positive test is also determined by spectrophotometric absorption detection of a transient color at about 2 minutes following the addition of the acid reagent, and preferably, the absorption is detected at a wavelength of about 620 nanometers. In preferred specific embodiments, the absorption detected at about 620 nanometers comprises a first absorption at a reaction time, wherein the first absorption comprises correction for interference by subtracting a second absorption at a different wavelength, or by subtracting a second absorption at a different reaction time.

[0021] In another embodiment of the present invention, screening a mammal for a genetic disorder of sterol metabolism is performed by a method further comprising the step of drying the sterol extract with a solid drying agent. In a preferred specific embodiment, the solid drying agent is sodium sulfate.

[0022] Also within the scope of the invention, screening a mammal for a genetic disorder of sterol metabolism is performed by a method further comprising the step of removing solid particles from the test sample, the sterol extract, or the reaction mixture. In a specific embodiment, the step of removing comprises filtration or centrifugation.

[0023] In another embodiment of the present invention, screening a human for Smith-Lemli-Opitz syndrome is performed by a method comprising the steps of collecting a test sample from the human to be tested; extracting the sterols from the test sample, concentrating the sterol extract, forming a reaction mixture by mixing the concentrated sterol extract or a solution of the concentrated sterol extract with an acid reagent, and comparing a transient color of the reaction mixture with a transient color of a control mixture to determine a positive test for Smith-Lemli-Opitz syndrome.

[0024] In specific embodiments, the test sample is selected from the group consisting of a dried blood spot and a body fluid. The dried blood spot is generally on a Guthrie card, and preferably on a Guthrie card that has been treated with a chemical stabilizer.

[0025] In preferred specific embodiments, the chemical stabilizer slows, delays, or prevents decomposition of 7-dehydrocholesterol and 8-dehydrocholesterol. A chemical stabilizer is selected from a group consisting of butylated hydroxytoluene (BHT), alpha-tocopherol (vitamin E), N-acetyl cysteine, tert-butyl phenyl nitrone, ascorbic acid (vitamin C), mannitol, and beta-carotene.

[0026] In specific embodiments, the extracting of the test sample comprises the steps of adding to the test sample about 16 volumes of methanol, about 30 to 50 volumes of chloroform, and stirring for about 30 minutes or mixing on a vortex apparatus for about 1 minute.

[0027] In specific embodiments of the present invention, the sterol extract is concentrated by removing liquid.

[0028] In further specific embodiments, the acid reagent is Liebermann-Burchard reagent, and preferably, the Liebermann-Burchard reagent is modified by diluting with chloroform. Also within the scope of the present invention, the acid reagent comprises triflic acid or trifluoromethanesulfonic acid at about 0.5% final volume. Further, the acid reagent comprises sulfuric acid in the range of about 0.2% to about 8% final volume. In another preferred specific embodiment, the acid reagent comprises a superacid.

[0029] In other specific embodiments, the concentrated sterol extract or a solution of the concentrated sterol extract is at a first subambient temperature and the acid reagent, which is at a second subambient temperature, are mixed. In another specific embodiment, the mixing comprises a stopped-flow cell apparatus, and preferably, the stopped-flow cell apparatus is cooled to a subambient temperature.

[0030] In specific embodiments, the comparing of the reaction mixture to a control mixture comprises visualization of a transient color at about 5 seconds following the addition of the acid reagent, wherein the visualization of a transient pink color indicates a positive test for Smith-Lemli-Opitz syndrome.

[0031] In one specific embodiment, a spectrophotometer comprises a diode array detector. The spectrophotometer detects absorption of the reaction mixture at about 5 seconds following the addition of the acid reagent at a wavelength of about 510 nanometers. The time of about 5 seconds corresponds to the maximum absorbance associated with the transient color.

[0032] In preferred specific embodiments, the absorption of a transient color at about 5 seconds following the addition of the acid reagent, wherein an absorbance at a first wavelength of about 510 nanometers corrected for interference by subtracting an absorbance at a second wavelength of about 560 nanometers is greater than about 0.050 AU indicates a positive test for Smith-Lemli-Opitz syndrome. Further, the absorption at a wavelength of about 620 nm, wherein the absorption is corrected for interference by subtracting a second absorption at a second wavelength, or by subtracting a second absorption at said wavelength at a second reaction time. Preferably, spectrophotometric absorption detection at about 620 nanometers determined at about 2 minutes compared to absorption at about 30 minutes following the addition of the acid reagent gives the ratio greater than about 0.1 indicates a positive test for Smith-Lemli-Opitz syndrome.

[0033] In another embodiment of the present invention, a human is diagnosed with Smith-Lemli-Opitz syndrome comprising the steps of collecting a test sample from the human to be tested, extracting sterols from the test sample, concentrating the sterol extract, forming a solution by dissolving the concentrated sterol extract with a deuterated organic solvent, and analyzing the solution by nuclear magnetic resonance analysis, and the presence of a singlet at about 0.620 ppm indicates the presence of 7-dehydrocholesterol and diagnoses Smith-Lemli-Opitz syndrome. In a specific embodiment, the nuclear magnetic resonance spectrometer is of at least about 300 MHz.

[0034] In one embodiment of the present invention, a human is diagnosed with Smith-Lemli-Opitz syndrome comprising the steps of collecting a test sample from the human to be tested, extracting sterols from the test sample, concentrating the sterol extract, forming a solution by dissolving said concentrated sterol extract with a deuterated organic solvent, and analyzing the solution by nuclear magnetic resonance analysis, and

the presence of a singlet at about 0.652 ppm indicates the presence of 8-dehydrocholesterol and diagnoses Smith-Lemli-Opitz syndrome.

[0035] In specific embodiments, the nuclear magnetic resonance spectrometer is of at least 300 MHz and comprises an automatic sample changer, a probe having a proton inner coil, and software for automated peak detection to provide high-throughput screening.

[0036] In another embodiment of the present invention, there is a method to screen a human for Smith-Lemli-Opitz syndrome further comprising the step of drying the sterol extract with a solid drying agent. In a preferred specific embodiment, the solid drying agent is sodium sulfate.

[0037] Also within the scope of the invention, there is a method to screen a human for Smith-Lemli-Opitz syndrome further comprising the step of removing solid particles from the test sample, the sterol extract, or the reaction mixture. In a preferred specific embodiment, the test sample, the sterol extract, or the reaction mixture is filtered. In another preferred specific embodiment, the test sample, the sterol extract, or the reaction mixture is centrifuged.

[0038] In one embodiment of the present invention, a human is diagnosed with Conradi-Hunermann syndrome comprising the steps of collecting a test sample from the human to be tested, extracting sterols from the test sample, concentrating the sterol extract, forming a solution by dissolving the concentrated sterol extract with a deuterated organic solvent, and analyzing the solution by nuclear magnetic resonance analysis, wherein the presence of a singlet at about 0.652 ppm diagnoses Conradi-Hunermann syndrome.

[0039] In one embodiment, there is a method to treat a Guthrie card comprising impregnating the Guthrie card with a chemical stabilizer. The chemical stabilizer retards, slows, delays, or prevents decomposition of a blood component, which preferably is a sterol. The chemical stabilizer is applied to the Guthrie card and preferably comprises butylated hydroxytoluene. The treated Guthrie card comprises a test sample that is screened and analyzed for a genetic disorder of sterol metabolism, which preferably is Smith-Lemli-Opitz syndrome.

[0040] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating

preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF SUMMARY OF THE DRAWINGS

[0041] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein:

[0042] FIGS. 1A and 1B. are the time course of transient color determined by spectrophotometric absorption at 620 nm of unsaturated sterols and acetate derivatives to the Liebermann-Burchard reagent.

[0043] FIGS. 2A and 2B illustrate the transient color response to Liebermann-Burchard determined by spectrophotometric absorption of 7DHC and cholesterol.

[0044] FIG. 3 shows a linearity plot of the spectrophotometric response of 7DHC acetate and oleate derivatives to the Liebermann-Burchard reagent.

[0045] FIG. 4 shows the spectrophotometric responses of SLOS and normal blood samples to modified Liebermann-Burchard reagent.

[0046] FIGS. 5A and 5B show the wavelength scan of clinical samples and controls for the detection of 7DHC.

[0047] FIG. 6 shows the wavelength scan of a 7DHC standard in chloroform.

[0048] FIG. 7 shows the transient color visualized in the colorimetric response of 7DHC standards to Liebermann-Burchard reagent.

[0049] FIG. 8 shows the transient color of 7DHC standards visualized at room temperature after one hour following reaction with modified Liebermann-Burchard reagent.

[0050] FIGS. 9A and 9B are spectrophotometric absorption measurements of 7DHC standard obtained after using a stopped-flow cell apparatus to mix the acid reagent with the 7DHC standard.

[0051] FIGS. 10A and 10B are the wavelength scans of 7DHC standards, representing a concentration range of 44 μg/mL to 0.7 μg/mL, following reaction with modified Liebermann-Burchard reagent.

[0052] FIG. 11A is the wavelength scan of a serum sample taken from a newborn suspected of having SLOS. FIG. 11B shows the visual transient color of the serum sample and 7DHC standards after reaction with acid reagent.

[0053] FIGS. 12A and 12B are the NMR spectra of reaction mixtures of SLOS blood and normal blood. Spectra includes the characteristic upfield region used to diagnosis SLOS.

[0054] FIGS. 13A through 13D are the NMR spectra of sterols from normal and varying degrees of SLOS blood samples.

[0055] FIGS. 14A through 14D are the NMR spectra of sterols from SLOS dried blood spots on Guthrie cards that had been treated with a chemical stabilizer.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

[0056] As used herein the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more.

[0057] As used herein, "subambient temperature" means a temperature that is below and not equal to an ambient temperature. In a preferred embodiment, the subambient temperature is achieved by contacting the outside of a container with ice, wherein the contents of the container are then at a temperature about equivalent to the ice, and wherein the contents of the container is an acid reagent. In a further preferred embodiment, the subambient temperature is achieved by contacting the outside of a container with dry ice, wherein the contents of the container are then at a temperature about equivalent to the dry ice, and wherein the contents of the container is a reaction mixture, a concentrated sterol extract or a solution of the concentrated sterol extract. A skilled artisan recognizes that additional components are used to achieve subambient temperatures including, but not limited to, a dry ice-ethanol slurry and a dry ice-acetone slurry.

[0058] As used herein, the term "sterol" and "sterols" refer to biosynthetic and synthetic compounds that posses a tetracyclic ring structure similar to that of cholesterol and a 3β-oxy group. The sterol(s) are free alcohols or esters. One skilled in the art recognizes that sterols found in mammals include, but are not limited to, cholesterol, lanosterol, 7-dehydrocholesterol, 8-dehydrocholesterol and unsaturated sterols, which are present as free alcohols or as esters.

[0059] As used herein, "body fluid" refers to fluids that originate from a body of a mammal such as whole blood, blood serum, blood plasma, red blood cells, feces, saliva, sweat, tears, and any components thereof.

[0060] The term "dried blood spot" as used herein refers to a blood that is in a solid state such as those obtained on a Guthrie card. The dried blood spot is easily dissolved in a volume of a solvent suitable for analysis. A variety of solvents are known in the art. An example includes chloroform, and for nuclear magnetic resonance spectral analysis a deuterated organic solvent such as deuterated chloroform is used. Other deuterated and non-deuterated solvents are used, and one skilled in the relevant art knows the determinants for choosing a solvent for analysis, which include such determinants as sample solubility and solvent absorbance maximum.

[0061] As used herein, "a solution" refers to a solid compound, sample, or residue that is dissolved in a suitable solvent.

[0062] As used herein, "spectrophotometry" or "absorption spectrometery" refers to the measurement of the absorption or transmission of incident light through solutions of test samples, wherein the test sample comprises an analyte of interest. Typically, analytes of interest have characteristic spectra, transmitting or absorbing specific wavelengths of light, which can be used to determine the presence of these analytes or measure their concentration in test samples. In the present invention, the analyte is a sterol, and preferably the sterol is cholesterol, 7DHC, 8DHC, or a combination thereof.

[0063] As used herein, "spectrophotometer" refers to an analytical instrument designed for spectrophotometric absorption having a light source, for which the emitted wavelength is known and is adjusted, and one or more detectors sensitive to desired wavelengths of transmitted or reflected light. In a preferred embodiment, the spectrophotometer comprises an absorption spectrophotometer. In a specific preferred embodiment, the spectrophotometer comprises a diode array detector. In another preferred embodiment, the spectrometer comprises a stopped-flow cell apparatus, wherein

the stopped-flow cell apparatus automates mixing of a concentrated sterol extract or a solution of the concentrated sterol extract with an acid reagent. In another specific embodiment, the spectrophotometer comprises an autosampler.

[0064] The term "spectrophotometric absorption" as used herein refers to measurement of the absorption (or, conversely, transmission) of incident light by colored analytes at specific wavelengths, irrespective of the state of polarization of the light. One skilled in the art recognizes that spectrophotometric absorption is also used to determine the amount of a given analyte that is present in a sample or reaction mixture.

[0065] The term "spectrophotometric absorption detection" as used herein means quantitative detection of analytes in a sample or reaction mixture by determining an absorption of light at various wavelengths, without regard to the state of polarization of the incident or absorbed light. Absorption in this case is proportional to the number of molecules of analyte present in a test sample or reaction mixture. In a specific embodiment, the wavelengths that are measured are 510 nm, 510 nm after correcting for interference, 620 nm, and 620 nm after correcting for interference. A skilled artisan is aware the an absorption unit is designated as AU.

[0066] "NMR spectrometer" as used herein refers to an analytical instrument that applies a magnetic field to a sample or reaction mixture, wherein the test sample or reaction mixture is subjected to an electrical radio frequency pulse applied to a coil surrounding the sample or reaction mixture. The pulse affects the nuclear magnetic spin of specific atoms in a molecule and produces a response called a "free induction decay", which results in an electrical signal that is detected, digitized and subjected to mathematical transformations. The result is a graph of peak intensity versus frequency. One skilled in the art is aware that a plot of the peak intensity versus frequency comprises an NMR spectrum.

[0067] As used herein, the term "colorimetric" means qualitative detection of an analyte in a test sample that absorbs in the visible frequencies and is related to a radiant energy source, such as a deuterium lamp, the chemical constitution of a sample or reaction mixture and/or of the analyte of interest, and the perception of the color observed.

[0068] As used herein, "superacid" refers to an acid that is stronger than 100 % sulfuric acid. It is known to a skilled artisan that superacids are used in chemical reactions to produce highly unstable and reactive intermediates such as organic

carbocations and reactive inorganic cations such as S_8^{2+}. Also, a skilled artisan recognizes that the conjugate base of a superacid is typically an inert anion.

[0069] As used herein, "chemical stabilizer" and "chemical stabilizers" refer to a chemical(s) that can slow the decomposition of biosynthetic or synthetic sterols, preferably during storage. These chemicals include, but are not limited to, butylated hydroxytoluene (BHT), alpha-tocopherol (vitamin E), N-acetyl cysteine, tert-butyl phenyl nitrone, ascorbic acid (vitamin C), mannitol, and beta-carotene. In a preferred embodiment, the chemical stabilizer slows, delays, or prevents the decomposition of the sample and components therein. In a preferred specific embodiment, the component is 7-dehydrocholesterol or 8-dehydrocholeserol.

[0070] As used herein, "impregnating" refers to permeating, filling, saturating, or imbuing a Guthrie card with a chemical stabilizer. The Guthrie card is preferably coated with the chemical stabilizer prior to placing a test sample, which is generally a blood spot, on the Guthrie card, which is accomplished by techniques known to one skilled in the art. Examples of the technique include overlaying the chemical stabilizer or a solution thereof onto the Guthrie card and producing filter paper that is prepared in the presence of the chemical stabilizer or a solution thereof.

II. The Present Invention

[0071] SLOS, a relatively common genetic disorder in populations of European heritage, is characterized biochemically by hypocholesterolemia and elevated 7DHC levels. Detection of 7DHC forms the basis of several methods for identifying affected individuals. The present invention encompasses a diagnostic test for SLOS based on differences in the qualitative and quantitative colorimetric response of cholesterol and 7DHC to the Liebermann-Burchard reagent. The claimed invention uses evaporated Folch extracts of human fluid samples. Mock samples containing 7DHC esters showed a brief initial absorbance at 510 nm (pink color), while cholesterol samples were initially colorless. After 2 min, the 7DHC samples absorbed strongly at 620 nm (blue color), compared to a weak absorbance for cholesterol samples. As incorporated herein, in blind tests normal individuals were distinguished from typical SLOS patients by visual monitoring of the initial transient pink color and by spectrophotometric measurements. Mild cases of SLOS could be tentatively identified by absorbances at 510-560 nm but not by visual observation or by the absorbance at 620 nm.

[0072] As incorporated herein, results were confirmed by nuclear magnetic resonance spectroscopy, which can distinguish mild cases of SLOS from normal subjects by determination of individual noncholesterol sterols. Because of its simplicity, the described colorimetric and spectrophotometric assay is further suitable for population-based screening for SLOS.

[0073] In one embodiment of the present invention, a mammal is screened for a genetic disorder of sterol metabolism comprising the steps of collecting a test sample from the mammal to be tested, extracting the sterols, concentrating the sterol extract, forming a reaction mixture by mixing the concentrated sterol extract or solution of the concentrated sterol extract with an acid reagent, and comparing a transient color of the reaction mixture with a transient color of a control mixture to determine the presence of a genetic disorder of sterol metabolism.

[0074] In specific embodiments, the test sample is selected from the group consisting of a dried blood spot and a body fluid. The dried blood spot is generally on a Guthrie card, and preferably the Guthrie card has been treated with a chemical stabilizer. In a preferred embodiment, the chemical stabilizer on the Guthrie card retards, slows, delays, or prevents the decomposition of 7-dehydrocholesterol. In another preferred specific embodiment, the chemical stabilizer is selected from a group consisting of butylated hydroxytoluene (BHT), alpha-tocopherol (vitamin E), N-acetyl cysteine, tert-butyl phenyl nitrone, ascorbic acid (vitamin C), mannitol, and beta-carotene.

[0075] In specific embodiments, the genetic disorder is selected from the group consisting of Smith-Lemli-Opitz syndrome, Conradi-Hunermann syndrome, CHILD syndrome, desmosterolosis, and Greenberg dysplasia.

[0076] In another specific embodiment, the extracting of the sterol from the test sample comprises the steps of adding to the test sample about 16 volumes of methanol, about 30 to 50 volumes of chloroform, and stirring for about 30 minutes or mixing on a vortex apparatus for about 1 minute.

[0077] In specific embodiments, the concentrating comprises removing liquid from the sterol extract. The removal of liquid can be done by techniques known in the art such as flowing a gas over the top of the liquid. Examples of a gas that one with ordinary skill in the art is aware for the purpose of concentrating a liquid sample include, but are not limited to, nitrogen, air, and argon. Also, vacuum evaporation is known by a skilled artisan to effectively concentrate a liquid sample or extract to a solid residue. Other

techniques are used to concentrate a liquid sample, and those techniques are known to those of ordinary skill in the art.

[0078] In specific embodiments, the acid reagent is Liebermann-Burchard reagent, and preferably the Liebermann-Burchard reagent is modified by diluting with chloroform.

[0079] In further specific embodiments, the concentrated sterol extract or a solution of the concentrated sterol extract is at a first subambient temperature and the acid reagent, which is at a second subambient temperature are mixed. In the present invention, mixing is performed by a stopped-flow cell apparatus, preferably the apparatus is cooled to a subambient temperature.

[0080] In specific embodiments of the present invention, a positive test for a genetic disorder of sterol metabolism is determined by visualization of a transient color at about 5 seconds following the addition of the acid reagent. Also within the scope of the invention is determining a positive test by spectrophotometric absorption of a transient color at about 2 minutes following the addition of the acid reagent, and preferably the absorption is detected at a wavelength of about 620 nanometers. In preferred specific embodiments, the first absorption at a first wavelength of about 620 nanometers is corrected for interference by subtracting a second absorption at a second wavelength, or by subtracting a second absorption at a second reaction time.

[0081] In further embodiments, there is a step of drying the sterol extract with a solid drying agent. The solid drying agent is selected from the group consisting of sodium sulfate, magnesium sulfate, calcium chloride, calcium sulfate, silica gel, and molecular sieves and preferably is sodium sulfate.

[0082] In a further embodiment, a mammal is screened for a genetic disorder of sterol metabolism by a method further comprising the step of removing solid particles from the test sample, the sterol extract, or the reaction mixture. In specific embodiments, the solid particles are removed by filtration. One skilled in the art is aware that several suitable filtration systems are used to remove solid particles from a solution. Examples of suitable filtration systems include, but are not limited to, filter paper and a conical funnel, filter paper and a Buchner funnel, and glass wool plugs. The solid particles are also removed by centrifugation, which a skilled artisan recognizes that centrifugation speed and duration are optimized to remove a solid relative to its respective density.

[0083] In another embodiment of the present invention, a human is screened for Smith-Lemli-Opitz syndrome by the method comprising the steps of collecting a test sample from the human to be tested, extracting the sterols from the test sample, concentrating the sterol extract, forming a reaction mixture by mixing the concentrated sterol extract or a solution of the concentrated sterol extract with an acid reagent, and comparing a transient color of said reaction mixture with a transient color of a control mixture to determine a positive test for Smith-Lemli-Opitz syndrome.

[0084] In specific embodiments, the test sample is selected from the group consisting of a dried blood spot and a body fluid. The dried blood spot is generally on a Guthrie card, and preferably the Guthrie card has been treated with a chemical stabilizer that slows, delays, or prevents decomposition of 7-dehydrocholesterol.

[0085] In a preferred specific embodiment, the chemical stabilizer on the Guthrie card is selected from a group consisting of butylated hydroxytoluene (BHT), alpha-tocopherol (vitamin E), N-acetyl cysteine, tert-butyl phenyl nitrone, ascorbic acid (vitamin C), mannitol, and beta-carotene.

[0086] In specific embodiments, the extracting of the sterols from the test sample comprises the steps of adding to the test sample about 16 volumes of methanol, about 30 to 50 volumes of chloroform, and stirring for about 30 minutes or mixing on a vortex apparatus for about 1 minute.

[0087] In specific embodiments, the concentrating comprises removing liquid from the sterol extract, and preferably the removing comprises flowing a gas over the top of the liquid or vacuum evaporation.

[0088] In specific embodiments of the present invention, the acid reagent is Liebermann-Burchard reagent, and preferably, the Liebermann-Burchard reagent is modified by diluting with chloroform. Also within the scope of the invention, the acid reagent comprises triflic acid or trifluoromethanesulfonic acid at about 0.5% final volume. In further specific embodiments, the acid reagent comprises sulfuric acid in the range of about 0.2% to about 8% final volume. The acid reagent comprises a superacid. In practicing the claimed invention, the concentration of the acid in the acid reagent is determined by comparing a reaction mixture comprising the concentrated sterol extract or a solution of the concentrated sterol extract with a control mixture.

[0089] In specific embodiments, the concentrated sterol extract or a solution of the concentrated sterol extract is at a first subambient temperature and the acid reagent,

which is at a second subambient temperature, are mixed. The mixing comprises a stopped-flow cell apparatus, and preferably the apparatus is cooled to a subambient temperature.

[0090] In specific embodiments, the comparing comprises visualization of a transient color at about 5 seconds following the addition of the acid reagent, wherein the visualization of a transient pink color indicates a positive test for Smith-Lemli-Opitz syndrome. In specific embodiments, the transient pink color indicates the presence of 7-dehydrocholesterol at a concentration of at least 70 μg/mL. In further specific embodiments, the transient pink color of the reaction mixture at a subambient temperature indicates the presence of 7-dehydrocholesterol at a concentration of at least 10 μg/ml.

[0091] In specific embodiments, the transient color of the reaction mixture is determined by a spectrophotometer comprising a diode array detector. A maximum absorbance detected by the spectrophotometer of a transient color at about 5 seconds following the addition of the acid reagent at a wavelength of about 510 nanometers.

[0092] In specific embodiments of the present invention, spectrophotometric absorption detection of a transient color at about 5 seconds following the addition of the acid reagent at a first wavelength of about 510 nanometers is corrected for interference by subtracting an absorbance at a second wavelength of about 560 nanometers. A corrected absorbance greater than about 0.050 AU indicates a positive test for Smith-Lemli-Opitz syndrome.

[0093] In further specific embodiments, a first spectrophotometric absorption detection at a wavelength of about 620 nm at a first reaction time corrected for interference by subtracting a second absorption at a different wavelength, or by subtracting a second absorption at said 620 nanometers at a second reaction time comprises comparing a reaction mixture to a control mixture. Preferably, .the spectrophotometric absorption detection at about 620 nanometers is determined at about 2 minutes compared to spectrophotometric absorption at about 30 minutes following the addition of the acid reagent, wherein the ratio of the first absorbance to the second absorbance is greater than about 0.1 indicates a positive test for Smith-Lemli-Opitz syndrome.

[0094] One skilled in the art is aware that correcting for interference of a first absorption at a first reaction time at a first wavelength includes subtracting a second absorption at a second wavelength, or subtracting a second absorption at a second

reaction time. A skilled artisan recognizes that corrections for interference improve sensitivity by increasing the signal-to-noise ratio and does not adversely affect real-time analysis.

[0095] In embodiments of the present invention, a human with Smith-Lemli-Opitz syndrome is diagnosed by a method comprising the steps of collecting a test sample from the human to be tested, extracting sterols from the test sample, concentrating a sterol extract, forming a solution by dissolving said concentrated sterol extract in a deuterated organic solvent, and analyzing the solution by nuclear magnetic resonance, wherein the presence of a singlet at about 0.620 ppm indicates the presence of 7-dehydrocholesterol diagnoses Smith-Lemli-Opitz syndrome. Further, the presence of the singlet indicates the presence of 7-dehydrocholesterol at a concentration of at least 15 □g/ml.

[0096] In specific embodiments, the analysis comprises a nuclear magnetic resonance spectrometer of at least about 300 MHz, and the reaction mixture comprises deuterated chloroform, which preferably the deuterated chloroform comprises tetramethylsilane in about 0.05% final volume.

[0097] In preferred specific embodiments, nuclear magnetic resonance analysis is proton (^{1}H) analysis and comprises an inverse detection probe. One skilled in the art recognizes that sensitivity is optimized by varying acquisition times, interpulse delays, and tip angle. In preferred specific embodiments, the NMR conditions comprise an acquisition time of at least 1 second, and preferably comprise Fourier transformation, wherein the resulting Fourier transform spectrum is processed with a very mild Gaussian apodization window to produce a spectrum. Also within the scope of the present invention, the NMR spectrometer comprises an automatic sample changer and software for automated peak detection to provide high-throughput screening.

[0098] In one embodiment, a human is diagnosed with Smith-Lemli-Opitz syndrome by a method comprising the steps of comprising the steps of collecting a test sample from the human to be tested, extracting sterols from the test sample, concentrating a sterol extract, forming a solution by dissolving said concentrated sterol extract in a deuterated organic solvent, and analyzing the solution by nuclear magnetic resonance, wherein the presence of a singlet at about 0.652 ppm indicates the presence of 8-dehydrocholesterol and diagnoses Smith-Lemli-Opitz syndrome. In further specific embodiments, the presence of a singlet indicates the presence of 8-dehydrocholesterol at a concentration of at least 0.5 μg/ml.

[0099] In specific embodiments, the analysis comprises a nuclear magnetic resonance spectrometer of at least about 300 MHz, and the reaction mixture comprises deuterated chloroform, which preferably the deuterated chloroform comprises tetramethylsilane in about 0.02% final volume.

[00100] In preferred specific embodiments, nuclear magnetic resonance analysis is proton (^1H) analysis and comprises an inverse detection probe. One skilled in the art recognizes that sensitivity is optimized by varying acquisition times, interpulse delays, and tip angle. In preferred specific embodiments, the nuclear magnetic resonance spectrometer conditions comprise an acquisition time of at least 1 second, and preferably comprise Fourier transformation, wherein the resulting Fourier transform spectrum is processed with a very mild Gaussian apodization window to produce a spectrum. Also within the scope of the present invention, the nuclear magnetic resonance spectrometer comprises an automatic sample changer and software for automated peak detection to provide high-throughput screening.

[00101] Also within the scope of the invention, the generated nuclear magnetic resonance spectrum is examined for the intensity of signals at 0.620 ppm and 0.652 ppm, optionally in comparison with the intensity of the signal at 0.679, wherein the signal at 0.679 ppm is the resonance of C-13 methyl of cholesterol.

[00102] In embodiments of the invention, the method to screen a human for Smith-Lemli-Opitz syndrome further comprises the step of drying the sterol extract with a solid drying agent, and preferably the solid drying agent is sodium sulfate. One skilled in the relevant art recognizes that a drying agent suitable for drying, *i.e.*, removing hydrophilic molecules, of solutions that wholly or primarily comprise an organic solvent include, but are not limited to, sodium sulfate, magnesium sulfate, calcium chloride, calcium sulfate, silica gel, and molecular sieves. Further, a skilled artisan knows that the drying agent is preferably anhydrous in order to remove efficiently the water from the organic solution.

[00103] In further embodiments of the invention, the method to screen a human for Smith-Lemli-Opitz syndrome further comprises the step of removing solid particles from the test sample, the sterol extract, or the reaction mixture. The solid particles are removed preferably by filtration or centrifugation. One skilled in the art is aware that several suitable filtration systems are used to remove solid particles from a solution. Examples of suitable filtration systems include, but are not limited to, filter paper and a conical funnel, filter paper and a Buchner funnel, and glass wool plugs. A skilled artisan also recognizes

that centrifugation speed and duration are optimized to remove a solid relative to its respective density.

[00104] In one embodiment of the present invention, a human is diagnosed with Conradi-Hunermann syndrome by a method comprising the steps of collecting a test sample from the human to be tested, extracting sterols from the test sample, concentrating the sterol extract, forming a solution by mixing the concentrated sterol extract with a deuterated organic solvent, analyzing the solution by nuclear magnetic resonance analysis, and the presence of a singlet at about 0.652 ppm diagnoses Conradi-Hunermann syndrome.

[00105] In one embodiment, a Guthrie card is treated by a method comprising impregnating the Guthrie card with a chemical stabilizer. The chemical stabilizer retards, slows, delays, or prevents decomposition of a blood component, which preferably is a sterol. The chemical stabilizer is applied to the Guthrie card and is selected from a group consisting of butylated hydroxytoluene, ascorbic acid, alpha-tocopherol, beta-carotene, mannitol, tert-butyl phenyl nitrone and N-acetyl cysteine, and preferably comprises butylated hydroxytoluene. The treated Guthrie card comprises a test sample that is screened and analyzed for a genetic disorder of sterol metabolism, which preferably is Smith-Lemli-Opitz syndrome.

[00106] In the claimed invention, it is preferred that the spectrophotometric absorption detection is measured by spectrophotometric absorption at a wavelength at about 510 nm. Although the wavelengths selected may vary as the choices of reagents and conditions are changed, *e.g.*, volume of sample, volume of reagent, incubation temperature, incubation time, the actual values need not be specified since they are readily determinable by those of skill in the art. Generally, when, for example, Liebermann-Burchard reagent and the modified Liebermann-Burchard reagent are used, adequate wavelengths for the spectrophotometric absorption detection are at about 510 nm, about 510 nm corrected for interference by subtraction of absorption at about 560 nm, about 620 nm, and about 620 nm corrected for interference.

[00107] In one embodiment of the present invention, an acid reagent is mixed with a concentrated sterol extract or a solution of the concentrated sterol extract. In a specific embodiment, the acid reagent is Liebermann-Burchard reagent. In a preferred specific embodiment, the acid reagent is a modified Liebermann-Burchard reagent that has been diluted with chloroform. In another specific embodiment, the acid reagent is

Liebermann-Burchard reagent containing triflic acid or trifluoromethanesulfonic acid at about 0.5% final volume. In another specific embodiment, the acid reagent is Liebermann-Burchard reagent containing about 0.2 % to about 8 % sulfuric acid. Also in the scope of the invention is an acid reagent that comprises a superacid. One example of a superacid is H(CB11H6X6), wherein X is chlorine or bromine (Reed *et al.*, 2000).

[00108] In practicing the present invention, the rate at which the concentrated sterol extract or a solution of the concentrated sterol extract is mixed with the acid reagent is controlled, *i.e.*, increased or decreased, by incorporating a stopped-flow cell apparatus. The stopped-flow cell apparatus allows rapid mixing of the concentrated sterol extract and the acid reagent. It is within the scope of the claimed invention that the stopped-flow cell apparatus operate at a subambient temperature.

EXAMPLES

[00109] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those skilled in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

EXAMPLE 1

Relationship Between UV-Visible Absorption and Observed Color

[00110] The absorption spectra is given for a given wavelength (nm) and the respective color that is absorbed and visually observed.

Table I.

Absorption (nm)	Color absorbed	Color Observed
380-420	violet	green-yellow
420-440	violet-blue	Yellow
440-470	blue	Orange
470-500	blue-green	Red
500-520	green	Purple
520-550	yellow-green	Violet
550-580	yellow	violet-blue
580-620	red	blue-green
680-780	purple	Green

EXAMPLE 2

Colorimetric Responses of Individual Sterol Standards

[00111] To develop conditions for colorimetric testing of SLOS and normal blood samples, Liebermann-Burchard reagent was added to a series of sterol standards and the time course of the absorbances and color changes were determined (Table II and FIG. 1). For analytical purposes, the most useful absorbances were at 510 and 620 nm, which correspond to pink and blue colors, respectively. FIG. 1A shows the time course of cholesterol, 7DHC, 8DHC, and the $\Delta^{5,7,9(11)}$ sterol, which accumulate as free sterols and esters in SLOS (Ruan *et al.*, 2001). Most sterol standards were acetate derivatives, which have colorimetric properties similar to those of free sterols and fatty acid esters.

[00112] The colorimetric and spectrophotometric absorption response of additional unsaturated sterols that potentially are present in normal and SLOS blood were also evaluated (FIG. 1B and Table II). Marked differences were observed in the colorimetric responses of the individual sterols. 8DHC and the $\Delta^{6,8}$, $\Delta^{7,9(11)}$, $\Delta^{7,14}$, $\Delta^{8,14}$, $\Delta^{8,24}$, and

$\Delta^{5,7,9(11)}$ sterols displayed a blue color response immediately after addition of the reagent. The only compound other than 7DHC to produce an initial pink response was the $\Delta^{5,8(14)}$ sterol, which is present at negligible levels in SLOS and normal blood (Ruan *et al.*, 2001) and thus does not interfere with the colorimetric analysis of clinical samples. The $\Delta^{6,8(14)}$ sterol showed no color change during the first 30 min but gradually developed a pink color after 14 h. The 19-nor-$\Delta^{5,7,9}$ sterol had no colorimetric response to the Liebermann-Burchard reagent, even after several days.

Table II.

Sterol	Absorbance at 620 nm			Observed color changes						
	2 min	30 min	A_{max} (time)	3 s	20 s	2 min	10 min	30 min	2 h	48 h
1	0.04	1.0	1.0 (30 min)	colorless	*blue*	*greenish-blue*	green			
2	3.2	2.0	3.2 (1.8 min)	pink	blue	blue		*yellowish-green*		
3	1.0	1.2	1.4 (8 min)	blue		blue		*yellowish-green*		
4	1.4	1.6	1.9 (8 min)	pink		blue		*yellowish-green*		
5	0.3	0.8	0.8 (25 min)	blue			reddish-purple		*red*	
6	0.01	0.04	0.05 (9 min)	colorless			colorless		pink	*yellow*
7	2.1	1.5	2.1 (2 min)		blue	blue			*greenish-yellow*	
8	3.5	1.9	3.5 (1.8 min)	blue		blue			*yellowish-green*	
9	2.4	2.3	2.8 (11 min)	blue			blue		*yellowish-green*	
10	1.1	0.9	1.2 (6 min)	blue			blue		*yellowish-green*	
11	6.0	3.8	6.2 (1 min)	blue			*blue*		*yellowish-green*	
12	0.02	0.02	0.02	colorless					colorless	

[00113] The numerical designations correspond to the following sterols: **1**, cholesterol; **2**, 7-dehydrocholesterol acetate; **3**, 8-dehydrocholesterol; **4**, cholesta-5,8(14)-dien-3β-ol; **5**, 5α-cholesta-6,8-dien-3β-ol acetate; **6**, 5α-cholesta-6,8(14)-dien-3β-ol acetate; **7**, 5α-cholesta-7,9(11)-dien-3β-ol acetate; **8**, 5α-cholesta-7,9(11)-dien-3β-ol acetate; **9**, 5α-cholesta-7,14-dien-3β-ol acetate; **9**, 5α-cholesta-8,14-dien-3β-ol; **10**, 5α-cholesta-8,24-dien-3β-ol acetate; **11**, cholesta-5,7,9(11)-trien-3β-ol acetate; **12**, 19-norcholesta-5,7,9-trien-3β-ol acetate. All absorbances are given in AU/μmol.

[00114] Liebermann-Burchard reagent (2 ml) was added to sterol standards (50-200 μg powder), followed by vortex mixing for 15 s and spectrophotometric analysis. The column labeled A_{max} gives the magnitude and time of the maximum absorbance at 620 nm. The observed color changes are indicated for various times from 3 s to 48 h; italics indicate pale color, and underline denotes intense color.

EXAMPLE 3

Spectrophotometric Absorption Detection of 7DHC Standards

[00115] The cholesterol solution was initially colorless, gradually becoming pale blue, greenish blue, and finally green. By contrast, 7DHC acetate or oleate solutions showed an initial transient pink color, turned blue within 1 min, and gradually faded to pale yellowish green over the next 2 h. 8DHC and the $\Delta^{5,7,9(11)}$ acetate both showed an immediate blue color response to the Liebermann-Burchard reagent; this blue color subsequently darkened for the 8DHC but faded somewhat for the $\Delta^{5,7,9(11)}$ acetate. Spectrophotometric absorption measurements indicated a large difference between 7DHC and cholesterol absorbances (620 nm) at 2 min (FIG. 2A and 2B) and smaller differences at 30 min (Table II). The ratio of absorbances at 2 min and 30 min were also used to distinguish SLOS from normal blood samples. The colorimetric analyses described herein focus on the response of 7DHC; however, the results indicate that the absorbances of 8DHC and the $\Delta^{5,7,9(11)}$ sterol, also present in SLOS samples, do not interfere with the measurement of the initial 7DHC absorption at 510 nm and somewhat augment the 620 nm response for SLOS samples at 2 min.

[00116] FIG. 2A and 2B show colorimetric responses of 7DHC and cholesterol to the Liebermann-Burchard reagent. Times denote the start of scanning after mixing. Samples of 7DHC (60 μg in 0.6 ml chloroform, FIG. 2A) or cholesterol (300 μg in 0.6 ml chloroform, FIG. 2B) were mixed with the Liebermann-Burchard reagent (0.6 ml), followed by scanning from 800 nm to 280 nm at a fast scan rate (ca. 8 s per 100 nm); scanning was started at 600 nm for the first 7DHC spectrum. Spectra were started at the following times after mixing: 7DHC, 3 s (——), 10 s (- - -), 2 min (— - —), and 8 min (— - - —); cholesterol, 3 s (——), 2 min (- - -), 8 min (— - —), and 20 min (— - - —). Sterols were dissolved in chloroform to facilitate rapid mixing.

EXAMPLE 4

Linearity and Detection Limit of the Colorimetric Responses of 7DHC Esters

[00117] The linearity of the colorimetric response was investigated by measuring the absorbances at 620 nm for a series of concentrations of 7DHC acetate and 7DHC

oleate. To each tube containing 7DHC oleate (55-440 μg) or 7DHC acetate (3-367 μg) was added Liebermann-Burchard reagent (2 ml), followed by vortex mixing (15 s) and measurement of the absorbance at 620 nm after 1.75 min. As shown in FIG. 3, color responses were linear in the range of 0.15 to 1.5 AU. This range corresponds to 5-200 μg of 7DHC acetate or 100-200 μl of typical SLOS blood, which contains 50-800 μg/ml 7DHC, composed about equally of free sterol and fatty acid ester. As little as about 3 μg of 7DHC acetate is detected by visual observation of the initial transient pink color.

[00118] The nearly threefold difference in the slopes of the two lines is attributable to differences in formula weight between 7DHC acetate and 7DHC oleate, the low solubility of 7DHC oleate in the Liebermann-Burchard reagent, and the possibility of incomplete concentration of the 7DHC oleate sample.

EXAMPLE 5

Colorimetric Responses of SLOS and Normal Blood Samples

[00119] The results of colorimetric and spectrophotometric absorption assays of SLOS and normal blood samples are summarized in Table III. In initial experiments, plasma, serum, or red cell samples were saponified, and the evaporated hexane extract was subjected to the colorimetric screen. In these tests, SLOS samples were readily identified by their initial color and by their absorbance at 620 nm (FIG. 4). The SLOS samples (A and B) turned pink immediately after addition of the Liebermann-Burchard reagent and became blue within 1 min. In contrast, the normal sample (Z) showed no color change for the first 5 min, gradually becoming light yellowish green and finally green after 30 min. The ratios of absorbances (620 nm) at 30 min and 2 min were 1.2-2.3 for SLOS samples vs. 62 for the normal sample. Folch extracts of samples B and Z gave similar color changes and absorbance ratios (1.6 vs. 17). The Folch extraction is much simpler than the saponification procedure, and the screen of sterol standards, as described in Example 4, indicated that the 7DHC esters present in the Folch extract give color responses comparable to those of 7DHC.

[00120] A blind test of normal and SLOS blood was carried out on samples I-O and S-Y, which included several mildly affected SLOS individuals (J, L, N, and O). As shown in Table III, severe cases of SLOS (I, K, and M) were easily distinguished from normal subjects.

Table III

Sample	Type	A_{510} 5 s	$A_{510-560}$ 5 s	A_{620} 2 min	A_{620} 30 min	Color	1	2	3
SLOS blood samples									
H	B/F[a]			0.132	0.114	+	100	364	182
H	B/F[b]			0.424	0.400	+			
H	B/F[d]			0.501	0.359	+			
B	P/N[c]			0.225	0.518	+	1008	145	100
B	P/F[d]			0.242	0.379	+			
A	P/N[c]			0.270	0.445	+	809	134	99
B	R/N[c]			0.266	0.320	+	737	122	45
I	P/F	2.246	1.507	0.197	0.491	+	920	78	72
K	P/F	3.251	1.843	0.158	0.470	+	1170	77	69
M	P/F	1.805	0.830	0.108	0.476	+	1430	70	69
L	P/F	0.912	0.241	0.041	0.519	–	1490	17.5	8.4
J	P/F	1.018	0.263	0.043	0.476	–	1240	6.4	8.9
N	P/F	0.816	0.157	0.024	0.677	–	1860	9.5	10.2
O	P/F	1.298	0.253	0.079	0.833	–	1830	7.1	12.0
Normal blood samples									
S	P/F	0.483	0.149	0.040	0.555	–	1280	*	*
T	P/F	1.162	0.086	0.053	0.549	–	1490	*	*
U	P/F	1.708	0.088	0.016	0.616	–	1860	*	*
V	B/F[d]			0.060	0.487	–	**	**	**
W	B/F[d]			0.050	0.369	–	**	**	**
X	B/F			0.052	0.669	–	**	**	**
Y	B/F			0.050	0.623	–	**	**	**
Z	S/N			0.010	0.617	–	**	**	**
Z	S/F[d]			0.036	0.596	–			

[00121] Mild cases of SLOS could be tentatively identified by the corrected absorbance at 510 nm ($A_{510-560}$ in Table III) but not by visual observation, the absorbance at 620 nm, or the uncorrected absorbance at 510 nm. Another sample (H; not blinded) was clearly from a severely affected individual as determined by the intensity of the initial pink color and by the absorbance at 620 nm. The Folch extract from a 20-µl aliquot of sample H also produced a pink color and gave the distinctive absorbance pattern for SLOS.

[00122] Compounds 1, 2, and 3, for which concentrations (μg/ml) are provided, represent cholesterol, 7-dehydrocholesterol, and 8-dehydrocholesterol, respectively. The abbreviations for the type are as follows: B, whole blood; P, plasma; R, red cells; S, serum; F, Folch extraction; N, nonsaponifiable lipids; a, 20 microliters of blood product used; b, 50 microliters of blood product used; c, 80 microliters of blood product used; d, 200 microliters of blood product used; otherwise, 100 microliters of blood product was used.

[00123] Evaporated Folch extracts or nonsaponifiable lipids (NSL) were prepared from whole blood, plasma, or serum. After addition of 10 volumes of Liebermann-Burchard reagent (12.5 volumes for the 80-μl samples; 0.7 ml for the 20-50 μl samples), the initial color was observed visually (+ indicates pink color), and the absorbance was measured at 620 nm (A_{620}) after 2 min and 30 min. $A_{510\text{-}560}$ denotes the absorbance at 510 nm (A_{510}) after subtraction of the absorbance at 560 nm. Samples are listed in order of decreasing 7DHC levels.

[00124] Absorbances at 510 and 560 nm were determined using 50 μl of plasma by a modified procedure described in the Materials and Methods. Additional normal plasma samples showed the following values for $A_{510\text{-}560}$ and cholesterol concentration: 0.101 AU, 1640 μg/ml; 0.067 AU, 1950 μg/ml; 0.093 AU, 1550 μg/ml; 0.124 AU, 1750 μg/ml; 0.108 AU, 1930 μg/ml. Values at or below the detection limit of 0.5 μg/ml are indicated by a "*". Data not measured and known to be from normal individuals are indicated by an "**".

EXAMPLE 6

Colorimetric and Spectrophotometric Determination of SLOS Serum Samples

[00125] Serum samples from 8 SLOS patients and 8 controls were obtained and measurements for 7DHC were conducted in a blinded fashion. 4/8 SLOS samples were obtained from severe patients and 4/8 from patients with a mild phenotype. Lipids from the samples were obtained by Folch extraction prior to analysis. 7DHC was detected by a simple colorimetric reaction in which 7DHC produces a unique, intense color change. The color is detected visually and quantified with a spectrophotometer equipped with a stopped-flow cell apparatus, which allowed rapid mixing of the sample and the

Liebermann-Burchard reagent. Results of the colorimetric assay were compared to those obtained by GC-MS and NMR.

[00126] Using the colorimetric method, all control individuals were correctly identified (7DHC levels <0.15μg/ml by GC-MS). A clear diagnosis of SLOS was made visually in 4/4 (100%) of the samples from typically affected individuals (7DHC levels 60-128μg/ml by GC-MS). Using a spectrophotometric response obtained by a spectrophotometer equipped with a stopped-flow cell apparatus, a clear diagnosis of SLOS was made in samples from 8/8 (100%) of affected individuals (7DHC levels 2.3-128μg/ml by GC-MS) (FIG. 5A). FIG. 5A shows the wavelength scans of each of the four samples from mildly-affected individuals (2.3 – 7.1 μg/ml). The small peak at 510 nm clearly distinguishes each affected individual compared to the flat curve of the control sample (MS10863). The scan of sample MS10359 (73 μg/ml) is shown for comparison. Samples were determined to comprise the following concentrations of 7DHC: MS10863, 0.14 μg/ml, MS10362, 5.8 μg/ml, MS10798, 2.8 μg/ml, MS10587, 7.1 μg/ml, MS10083, 2.3 μg/ml, and MS10359, 73 μg/ml.

[00127] FIG. 5B shows the wavelength scans of each of four control samples (0.05 – 0.15 μg/ml). The flat curve in the region of 510 nm clearly distinguishes control samples from samples containing elevated 7DHC. Mildly affected sample MS10798 (2.8 μg/ml) and sample MS10359 (73 μg/ml) are shown for comparison. The amount of 7DHC present in the samples are MS10863, 0.14 μg/ml, MS10864, 0.15 μg/ml, MS10798, 2.8 μg/ml, MS10359, 73 μg/ml, and MS10826, 0.05 μg/ml. Using 7DHC chemical standards, the visual detection limit is approximately 10μg/ml 7DHC and the detection limit using a spectrophotometer equipped with a stopped-flow cell apparatus is approximately 0.1μg/ml 7DHC.

EXAMPLE 7

Transient Color Response of 7DHC

[00128] The transient color response of 7DHC to the Liebermann-Burchard reagent is visualized within 3 seconds from the start of the reaction (FIG. 6). The wavelength scan between 380 and 700 nm of a 7DHC standard (44 μg/ml in chloroform) at 2 second intervals revealed a nearly immediate response that is quantified by spectrophotometric

absorption detection. The absorption maximum decreases significantly within 10 seconds after adding Liebermann-Burchard reagent. The reaction time indicates that the colorimetric assay is amenable to modifications that allow a high-sample throughput.

EXAMPLE 8

Stabilizing the Transient Color Response

[00129] Because of the short life-time of the transient color observed in the reaction mixture of 7DHC and Liebermann-Burchard reagent, improvements in the reaction chemistry with the goal of stabilizing the pink intermediate at room temperature while maintaining or improving the specificity and sensitivity are made. By doing so, it is possible to achieve quantitative measurements of 7DHC and develop an automated means of testing samples. Modifications to the Liebermann-Burchard reagent which have been tested include systematic variation or elimination of acetic acid, acetic anhydride, and sulfuric acid from the reagent, augmentation of the reagent with high concentrations of sulfuric and superacids, systematic variation of reaction procedures and stoichiometry, and systematic testing of solvents. FIG. 7 shows the colorimetric response of stabilizing the transient pink color by placing the reaction mixture on dry ice. The approximate 7DHC concentrations are shown for each sample and are given in μg/ml. The observation that the intermediate generated when 7DHC is mixed with Liebermann-Burchard reagent indicates that a quantitative measure of 7DHC is feasible.

[00130] FIG. 8 shows the colorimetric response of stabilizing the pink intermediate at room temperature. The accurate concentration of 7DHC standard (μg/ml) in each sample is indicated in black text with the approximate value used during serial dilution drawn on each tube. 7DHC standard was dissolved in chloroform to a final concentration of 10,000 μg/ml, and serial dilution performed. Modified Liebermann-Burchard reagent was prepared by adding to the sample 1 microliter acetic anhydride followed by cooling to 0°C on ice water bath. To this solution was added 10 microliters of a cold (0 degrees C) mixture of 1 part triflic acid and 1 part sulfuric acid. The sample was mixed vigorously for 5 seconds and allowed to warm to room temperature, whereupon a stable pink color (A-maximum 510 nm) was seen to gradually increase in intensity and remain intense at room temperature for several hours. The samples were photographed at room temperature after 1 hour, and the color is stable indefinitely at –20°C.

EXAMPLE 9

Spectrophotometric Response Using A Stopped-Flow Cell Apparatus

[00131] Because the life time of the transient color is short, incorporating a stopped-flow cell apparatus to allow for rapid mixing of the sterol and the acid reagent was tested to increase the accuracy and precision of the spectrophotometric assay. 7DHC standard mixtures were tested in Liebermann-Burchard reagent (FIG. 9A) that comprises 2 % H_2SO_4, and a modified Liebermann-Burchard reagent (FIG. 9B) that comprises 16 % H_2SO_4. Triplicate measurements of 7DHC standard at 34 μg/ml using a KinTek stopped-flow cell apparatus. A stopped-flow rapid-mixing spectrophotometer allowed an accurate method of measuring the transient pink intermediate to a detection level as low as 0.1 μg/ml 7DHC. Systematic variation of reaction conditions and analysis by stopped-flow indicates that substantial improvements can be made to the Liebermann-Burchard reagent to improve sensitivity. FIG. 9B indicates that augmenting the Liebermann-Burchard reagent with 8 times the normal concentration of sulfuric acid more than doubled the signal intensity, effectively prolonging the existence of the pink color. These experiments indicate that certain elements of the reaction are limiting and that optimization of reaction chemistry greatly improves the utility of the method for clinical diagnosis.

EXAMPLE 10

Spectrophotometric Response of 7DCH Standards at Room Temperature

[00132] Stabilization of the pink intermediate at room temperature eliminates the need for kinetic analysis inherent to detection of a transient intermediate. To test the spectrophotometric absorption response of 7DHC standards to a modified Liebermann-Burchard reagent prepared by adding 1 microliter acetic anhydride to each standard followed by cooling to 0°C on ice water bath. To this solution was added 10 microliters of a cold (0 degrees C) mixture of 1 part triflic acid and 1 part sulfuric acid. The reaction mixture was mixed vigorously for 5 seconds and allowed to warm to room temperature, whereupon a stable pink color (A-maximum 510 nm) was seen to gradually increase in intensity and remain intense at room temperature for several hours. Wavelength scans of 7DHC standards at concentrations ranging from 44 μg/ml to 0.7 μg/ml were taken (FIG.

10A and 10B). Additional means to stabilize the transient pink intermediate include a robot mixing and performing the sample reading using a 96 well plate format.

[00133] FIG. 10 A shows the wavelength scans between 450 to 600 nm of 7DHC standards at concentrations of 44 (top), 22 (middle), and 11 (bottom) μg/ml. FIG. 10B shows the wavelength scans between 450 to 600 nm of 7DHC standards at concentrations of 2.8 (top), 1.4 (middle), and 0.7 (bottom) μg/mL.

EXAMPLE 11

Diagnosis of an Affected Newborn

[00134] Colorimetric detection of 7DHC in a serum sample from a newborn suspected of having SLOS was used to make the diagnosis of SLOS prior to clinical testing by GC-MS and NMR. The child was treated at Primary Children's Medical Center in Salt Lake City. The sample (PSPRG-01) was estimated to contain 50 μg/ml 7DHC based on the colorimetric and spectrophotometric assay. Independent analyses by GC-MS and NMR confirmed the diagnosis, indicating approximately 72 μg/ml 7DHC.

[00135] FIG. 11A shows the wavelength scans between 380 and 700 nm at 2 second time intervals of sample PSPRG-01, which clearly indicate the presence of elevated 7DHC by the strong absorbance maximum at 510 nm. FIG. 11B is a photograph of the transient color elicited by the sample and 7DHC standards with the concentration of the standards given above. Values shown are μg/ml 7DHC.

EXAMPLE 12

Triflic Acid as Acid Reagent

[00136] 7DHC is especially sensitive to a modified Liebermann-Burchard reagent containing triflic acid. An intense pink color is observed to be quantitative with respect to 7DHC concentration. The pink color is quantified at 510 nm and is stable at room temperature, allowing the pink color to be visualized and quantified at any time during a several hour period without need for special handling or cooling the reagent. A serum sample containing 7DHC is dissolved in chloroform at room temperature. 1 to 10 μL of acetic anhydride is added to the sample. 10 μL of a 1:1 mixture of concentrated sulfuric acid and triflic acid is added with mixing. The resulting intense pink color is then

quanitified by transferring the sample to a spectrophotometer cuvette and measuring the absorbance at 510 nm. The pink color is observed in a clear vessel when the concentration of 7DHC is greater than 2 μg/mL. The color change is quantified with the spectrophotometer when the concentration of 7DHC in the sample is greater than 0.1 μg/mL.

EXAMPLE 13

A Superacid as an Acid Reagent

[00137] The sensitivity of the reaction is further improved and the stability of the resulting colored species is improved by the addition of a new superacid (Reed *et al.*, 2000). As discussed herein, H(CB11H6X6) (where X = chlorine or bromine) is a new superacid whose conjugate base is the exceptionally inert CB11H6X6- carborane anion. This superacid displays many of the characteristics of triflic acid in the presence of 7DHC and is applicable to testing for 7DHC in blood samples.

EXAMPLE 14

NMR Analysis of Normal and SLOS Blood Samples

[00138] NMR analysis using a 5-10 min data acquisition on a Folch extract from 100 μl of blood reveals 7DHC and 8DHC signals representing 0.3% of total sterols (FIG. 12A and 12B). Characteristic methyl signals (H-18) for cholesterol, 7DHC, and 8DHC were well separated from each other at 500 MHz and, owing to their distinctive shielding (δ 0.6-0.7), rarely suffer interference from other substances, including lipid material in Folch extracts. FIG. 12A shows the upfield NMR spectrum of a sterol extract obtained from normal blood. The regions of the spectrum between 0.600 and 0.670 ppm are free of signals. FIG. 12B shows the upfield NMR spectrum of a sterol extract obtained from SLOS blood. The characteristic signals are labeled to designate the molecule to which the highly shielded methyl belongs. Because the ester and free sterol signals for H-18 in the Folch extract were only slightly resolved at 500 MHz, these signals could be quantitated together using appropriate apodization. Although NMR bears a high instrument cost and throughput limitations, it is a useful backup to more expedient methods and can eliminate false positive results, which arises from drug

treatment or interference from bilirubin, hemoglobin, creatine, or blood decomposition products.

EXAMPLE 15

NMR Analysis to Diagnose Mild SLOS

[00139] Blood from patients with varying degrees of severity of SLOS were analyzed by NMR after sterol extraction by saponification. FIG. 13A is a normal sample and was used as a negative control. FIG. 13B is the spectrum obtained from a patient with very mild SLOS, which is indicated by the low levels of Δ5,7 and Δ5,8 in the upfield region. FIG. 13C is the spectrum obtained from a patient with mild SLOS, and accordingly, the levels of Δ5,7 and Δ5,8 are increased relative to A and B. FIG. 13D is the spectrum obtained from a patient with severe SLOS as indicated by the major sterol signal belonging to Δ5,7 or 7DHC. The striking difference in the sterol profile in FIG. 13D compared to A through C indicates that severe SLOS is easily detected, but the more mild cases bear much less 7DHC and thus more difficult to detect. However, the results indicate that mildly affected SLOS individuals can be identified reliably from ^{1}H NMR spectra (FIG. 13A through 13D).

EXAMPLE 16

Decomposition of 7DHC and 8DHC in Dried Blood Spots

[00140] Neonatal blood is commonly archived on Guthrie cards as dried blood spots. In a blind test, blood from samples H (SLOS) and X (normal) was spotted on a Guthrie card and sent by overnight courier at ambient temperature for colorimetric and NMR analysis. Folch extraction was done on each sample, followed by NMR analysis and colorimetric testing. The NMR results indicated major decomposition of 7DHC and 8DHC in the SLOS sample, which showed a 4:1:1 ratio of cholesterol, 7DHC, and 8DHC instead of the expected 1:2:1 ratio. The SLOS sample gave an initial pink color, and the normal sample was initially colorless. The absorbances at 620 nm were as follows: SLOS sample, 0.338 AU at 2 min, 0.373 AU at 30 min; normal sample, 0.287 AU at 2 min, 0.600 AU at 30 min Although the SLOS sample could be correctly identified by the

color response and its absorbance at 620 nm, the normal sample would have been misidentified based on its high absorbance at 2 min. This absorbance potentially represents interference from decomposition of nonsterol blood components. Decomposition of 7DHC and 8DHC in SLOS blood spots on archived cards has been reported previously (Zimmerman *et al.*, 1997; Starck *et al.*, 2000).

EXAMPLE 17

Retarding Decomposition of 7DHC and 8DHC in Dried Blood Spots

[00141] As discussed herein, 7DHC decomposes over time in a dried blood spot. One means to stabilize the 7DHC is to optimize the storage conditions of the dried blood spots. To determine such optimal conditions, several storage conditions were tested. A sample of blood (30 μl) from an SLOS individual was spotted onto six individual circles of generic Guthrie cards (Schleicher & Schuell, catalog # 10538414). Two of these circles had been impregnated with a solution of butylated hydroxytoluene (BHT, 100 μg) in 20 microliters of ethanol, followed by evaporation of solvent). The filter paper circles were placed in a vial, and the blood was allowed to dry in loosely capped vials. These filter paper circles of dried blood spots were subjected to different storage conditions shown in the Table IV: After the specified length of time, the spotted circles were subjected to Folch extraction, followed by evaporation of solvent to dryness. The residue was analyzed by ^{1}H NMR at 500 MHz to determine the relative amounts of cholesterol and 7DHC. The cholesterol was assumed to be essentially stable, and the decomposition of 7DHC and 8DHC was measured by the deviation of the 7DHC:cholesterol or 8DHC:cholesterol ratio from those in undecomposed blood.

[00142] These results indicate that the following measures enhance the stability of 7DHC and 8DHC on Guthrie cards: treatment of the filter paper circle with BHT, storage of the dried blood spot in the dark, storage of the dried blood spot for a short (rather than long) time before analysis.

Table IV. Conditions for storage of Guthrie cards.

Time *days*	Light/Dark	BHT	7DHC *% decomposition*	8DHC *% decomposition*
1	light	no	60	47
1	light	yes	0	0
1	dark	no	6	5
4	light	no	88	86
4	dark	no	37	27
4	light	yes	11	07

EXAMPLE 18

NMR Analysis of BHT Treated Guthrie Cards

[00143] NMR analysis was performed on reaction mixtures that were derived from dried blood spots, obtained on Guthrie cards, treated with BHT. All blood spots were stored in light. FIGS. 14A and 14C show the upfield NMR spectrum of dried blood spots stored for 1 day and 4 days, respectively, with no BHT treatment. FIGS. 14B and 14D show the upfield NMR spectrum of dried blood spots stored for 1 day and 4 days, respectively, with BHT treatment. Using the cholesterol levels as an internal reference, a substantial difference in the amount of 7DHC and 8DHC are observed upon treatment with BHT. This data strongly indicates that stabilizing 7DHC and/ or 8DHC with a chemical stabilizer improves the accuracy of screening for genetic disorders of sterol metabolism, in particular SLOS, from a dried blood spot.

EXAMPLE 19

Stabilization of Sterols in Guthrie Cards by Treatment with Chemical Stabilizers

[00144] Retarding sterol decomposition is not limited to the use of BHT. A similar experiment is performed in which filter paper circles on Guthrie cards are treated with a chemical substance prior to contact of blood with the card. The purpose of this treatment

is to protect 7DHC, 8DHC, and their esters present in blood from decomposition on the Guthrie card. Otherwise, a large percentage of sterols decomposes during storage of Guthrie cards. This decomposition leads to an underestimation of the amount of 7DHC species in the blood samples and makes testing for Smith-Lemli-Opitz syndrome less sensitive. In one type of decomposition, 7DHC and its esters are oxidized to the 5α,8α-epidioxide derivative. Treatment of the Guthrie card with free radical scavengers or antioxidants, such as butylated hydroxytoluene, ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), beta-carotene, mannitol, tert-butyl phenyl nitrone, and *N*-acetyl cysteine protects against this kind of decomposition. This kind of decomposition is also mitigated by storage of the spotted Guthrie cards at subambient temperature. This kind of decomposition is further mitigated by storage of the spotted Guthrie cards in an oxygen-free atmosphere. In another type of decomposition, 7DHC undergoes photochemical cleavage by ultraviolet light to form secosterols. This kind of decomposition is mitigated by storage of the spotted Guthrie cards in the dark. Optimal mitigation of decomposition of 7DHC is achieved by a combination of these three measures, *i.e.* treatment of the Guthrie card with appropriate protective chemical substances, storage of the spotted Guthrie cards at subambient temperature, and storage of the Guthrie cards in the dark.

EXAMPLE 20

Screening for SLOS in Blood

[00145] A simple colorimetric assay that distinguishes SLOS from normal blood samples based on differences in levels of 7DHC is carried out on blood, plasma, serum, or red cell samples, which are subjected to the simple manipulations of Folch extraction, followed by evaporation of solvent and addition of Liebermann-Burchard reagent. SLOS samples give an immediate pink color (λ_{max} 510 nm) and gradually become blue over 1-2 min (λ_{max} 620 nm). By contrast, normal blood samples are initially colorless and become very faintly blue after 2 min. Visual observation of color is sufficient to distinguish typical SLOS cases from normal individuals, and spectrophotometric measurements at 510 and 560 nm permitted at least tentative identification of all SLOS plasma samples. Although the transient absorbance at 510 nm must be measured within seconds after mixing, the absence of interference from cholesterol at this early time allows detection of traces of 7DHC in the presence of cholesterol. Similar measurements at 620 nm are less

discriminating because the 7DHC response does not develop until ca. 2 min, at which time the response of cholesterol has become significant (FIG. 2).

EXAMPLE 21

A Colorimetric and Spectrophotometric Assay to Screen for SLOS

[00146] The colorimetric assay for SLOS has several merits relative to other diagnostic methods. The capital and maintenance costs of instrumentation are much lower than those required for mass spectral analyses (Zimmerman *et al.*, 1997; Sattler *et al.*, 1995). Sample preparation comprises an evaporated Folch extract which is much simpler than the saponification required for GC or GC/MS analyses (Kelley *et al.*, 1995; Tint *et al.*, 1994), and direct addition of Liebermann-Burchard reagent to serum, an automated method developed for the colorimetric assay of cholesterol (Zak, 1980; Tonks, *et al.*, 1967; Katan *et al.*, 1982), is feasible. Potential interference from nonsterol materials in blood has been studied extensively for similar colorimetric assays (Zak, 1980; Tonks, *et al.*, 1967; Katan *et al.*, 1982; Sommers, *et al.*, 1975) and thus is addressed more easily than in direct UV measurement of 7DHC levels at 280 nm (Honda *et al.*, 1997). Colorimetric testing, both qualitatively and quantitatively, is faster and simpler than cell culture assays (Honda *et al.*, 1998; Lund *et al.*, 1996), which require lengthy incubations, and more universal than PCR methods (Yu *et al.*, 2000; Battaile *et al.*, 1999), which detect only specific mutations. These desirable features of colorimetric testing indicate its potential value in universal screening for SLOS.

EXAMPLE 22

Large-Scale Screening for SLOS

[00147] Large-scale screening would require a diagnostic test that discriminates mild SLOS cases from SLOS carriers and normal subjects. Plasma cholesterol levels in mildly affected SLOS individuals are ca. 1000-2000 μg/ml, compared to 5-10 μg/ml 7DHC. For samples reflecting these drastic differences in cholesterol and 7DHC levels, interference was encountered from cholesterol in measuring the colorimetric response of 7DHC at 620 nm. Interference by cholesterol might also occur in mass spectral screening methods because unsaturated sterols show traces of M-2 ions that appear to originate

from dehydrogenation in the ion source (Gerst *et al.*, 1997). Further problems arise from substantial decomposition of the 7DHC analyte on Guthrie cards (Starck *et al.*, 2000) and from interference by biological materials present at trace levels. However, interference from drugs, a potential problem in SLOS children (Nissinen *et al.*, 2000), should be minimal in neonatal screening. At its present stage of development, the colorimetric method can tentatively identify mildly affected SLOS individuals. Procedural refinements, such as use of a special mixing device, carrying out measurements at controlled subambient temperatures, or modifying the composition of the acid reagent lead to even better discrimination between SLOS and normal individuals. The distinctive color response of 7DHC to the Liebermann-Burchard reagent has been developed into a method for identifying SLOS individuals. The test requires small amounts of human body fluid, limited sample processing, inexpensive reagents, and simple, low-maintenance instrumentation. Positive test results are confirmed by NMR, which provides unequivocal identification of the noncholesterol sterols that uniquely characterize SLOS. Attempts to improve the colorimetric test to permit more definitive detection of mild cases of SLOS, which likely escape clinical detection and timely treatment. Variations of this test are useful for screening of additional genetic disorders of cholesterol synthesis.

EXAMPLE 23

GC and GC-MS Methods for Sterols

[00148] Capillary GC was carried out on a Shimadzu GC-9A instrument with a 30-m DB-5 column (0.25 mm i.d.; 0.1 μm film thickness; J&W Scientific; Folsom, CA) at 250 °C with nitrogen carrier gas at 1.1 kg/cm^2. Additional GC analyses were done on a Perkin Elmer Sigma 2000 chromatograph with split injection using a 60-m DB-5 column (0.25 mm i.d.; 0.1 μm film thickness) at 250 °C with nitrogen carrier gas at 1.3 kg/cm^2. GC-MS was done on an HP-5890A GC unit containing a 60-m DB-5ms column (0.25 mm i.d.; 0.1 μm film thickness; 250°C; helium carrier gas at 1.4 kg/cm^2) interfaced to a ZAB-HF mass spectrometer containing an electron-impact ion source (70 eV; 200°C). Mass spectral data are presented as *m/z* (relative abundance). Steryl acetates were analyzed directly by GC after addition of F_7-cholesterol acetate and/or 5α-cholestane.

Samples of free sterols with corresponding internal standards were converted to TMS derivatives by treatment with a 1:1 mixture (200 µl) of *N,O*-bis(trimethylsilyl)trifluoroacetamide and pyridine at 50 °C for 2 h.

[00149] Individual sterols were quantitated by GC and GC-MS against known amounts of internal standards (5α-cholestane; F_7-cholesterol acetate or TMS derivative), after correction for differences in detector response and the increased mass arising from derivatization. The relative responses (based on mass injected) of the acetates of F_7-cholesterol, Δ^5, Δ^7, $\Delta^{5,7}$, and $\Delta^{5,8}$ and of 5α-cholestane to flame ionization detectors were 1.0, 1.7, 1.7, 1.7, 1.6, and 1.8, respectively. These sterols showed linear detector responses with 30-m and 60-m DB-5ms columns over the range of 3-150 ng, although quantitation of the broad $\Delta^{5,7}$ TMS or acetate peak was sometimes imprecise. Relative amounts of sterols in mixtures were also measured by ^{1}H NMR, mainly by comparison of the relative intensities of the upfield methyl signals (δ 0.5-1.1) after slight Gaussian apodization (LIEBERMANN-BURCHARD –0.8, GB 0.08, 9k to 16k complex points). Acquisition times for trace-level analyses were typically 1.5 s, with a ~70° pulse and a 10-ms relaxation delay. T_1 relaxation times for the H-18 and H-19 signals of representative sterols in nondegassed $CDCl_3$ were both ~0.7 s.

EXAMPLE 24

Method of Assaying with Liebermann-Burchard Reagent

[00150] Colorimetric assays of sterol standards were carried out by adding 2 ml Liebermann-Burchard reagent to 50-200 µg of a sterol in a 7-ml tube, followed by vortex mixing for 15 seconds. A portion of the resulting solution was transferred to a quartz cuvette for spectrophotometric analysis at 25°C. Spectra (300-800 nm) were measured by photodiode array every 15 seconds for at least 40 minutes, and the absorbance at 620 nm was plotted versus time. The color response was also observed visually.

[00151] Plasma, red cells, serum, or whole blood was subjected to either saponification or Folch extraction. Saponification was done by heating the sample for 2 h with 15% ethanolic KOH, followed by hexane extraction and evaporation to dryness. In the Folch extractions, serum, plasma, or blood was added dropwise with stirring to 16 volumes of methanol, and the solution was stirred for 5 min (or vortexed for 2 x 20 s). Then chloroform (30-50 volumes) was added, followed by stirring for 30 min (or

vortexing for 2 x 20 s), optional drying over anhydrous Na_2SO_4, filtration or centrifugation to remove solids, and thorough evaporation of the solvent to a white or yellowish residue. To the residue was added 1 ml of Liebermann-Burchard reagent for each 100 µl of plasma, serum, or blood (or 100 mg of red blood cells). The initial color was observed visually, and the absorbance was measured either at 620 nm after 2 min and 30 min or at 510-560 nm after 5 seconds. Traces of hydroxylic solvent, such as ethanol, which is present as a preservative in chloroform, suppressed the color response.

[00152] The Liebermann-Burchard color reagent (Abell *et al.*, 1952) was prepared freshly by adding concentrated H_2SO_4 (1 ml) to acetic anhydride (20 ml) at 0°C, followed by stirring for 10 min, addition of acetic acid (10 ml), and warming to room temperature before use.

EXAMPLE 25

Method of Assaying with Modified Liebermann-Burchard Reagent

[00153] A modified procedure was used for measuring absorbances at 510 nm on SLOS and normal plasma samples. The standard Liebermann-Burchard reagent (5 ml) was diluted with an equal volume of chloroform and cooled to 0°C, followed by addition of 1.5 ml of concentrated H_2SO_4. An evaporated Folch extract from 50 µl of normal or SLOS plasma was dissolved in 50 µl of chloroform and chilled to -78°C in an Eppendorf tube. To this solution was added 50 µl of the cold modified Liebermann-Burchard reagent. The combined solution was rapidly drawn in and out of the pipet three times and then injected into a microcell (at ambient temperature) of a Beckman DU 7400 diode array spectrophotometer, which was already set to collect spectra every 3 seconds. The time elapsed from initial mixing to data collection was ca. 5 seconds Because the initial absorbance at 510 nm correlated poorly with 7DHC levels, correction for unidentified interferences (and variations in the time and temperature of the measurements) was made by subtraction of the absorbance at 560 nm.

[00154] Liebermann-Burchard reagent was also modified by increasing the sulfuric acid composition to 16 % final volume (FIG.9B). A more intense pink color was observed indicating that the concentration of sulfuric acid in the Liebermann-Burchard reagent (normally 2%, FIG. 9A) is a variable parameter which contributes to the accuracy of the assay for Smith-Lemli-Opitz.

EXAMPLE 26

Spectral Methods

[00155] Spectrophotometric absorption analysis of Liebermann-Burchard reactions was done in 1.4- or 3.5-ml quartz cuvettes (10-mm path length) on the following instruments: Hewlett Packard 89500 photodiode-array instrument (samples A, B, and Z and authentic sterol standards; cell thermostatted at 25°C), Shimadzu 1601 spectrophotometer (other blood samples; kinetic studies with cholesterol and 7DHC), and Varian Series 634 spectrophotometer (linearity studies). In addition, plasma absorbances at 510 nm were measured on a Beckman DU 7400 diode array spectrophotometer using a quartz 50 μl microcell. ^{1}H nuclear magnetic resonance (NMR) spectra were acquired at 25°C on a Bruker AMX-500 spectrometer and referenced to internal tetramethylsilane; solutions were ≤5 mM in $CDCl_3$. Mass spectra were obtained by direct probe on a ZAB-HF spectrometer with electron-impact ionization (70 eV).

EXAMPLE 27

Synthesis of 7-dehydrocholesteryl oleate

[00156] To a solution of 7-dehydrocholesterol (600 mg, 1.56 mmol) and oleic acid (700 mg, 2.5 mmol) in dichloromethane (10 ml) at 0°C was added a solution of dicyclohexylcarbodiimide (500 mg, 2.4 mmol) and 4-(dimethylamino)pyridine (50 mg) in methylene chloride (10 ml). The resulting mixture was warmed to room temperature and stirred for 24 h. The reaction was diluted with hexane (200 ml), and the precipitated dicyclohexylurea was removed by filtration. The filtrate was washed with 5% H_2SO_4 (50 ml), 5% $NaHCO_3$ (50 ml), and brine (50 ml), followed by filtration through neutral alumina (8 cm x 2 cm column; elution with hexane). Evaporation of the eluate to dryness under reduced pressure gave an oily residue. Recrystallization from ethanol furnished 7-dehydrocholesteryl oleate (639 mg; 63% yield) as a waxy solid: single component on TLC (R_f 0.9; acetone-hexane 2:8); MS, *m/z* 648 (2, M^+), 366 (100, M-oleic acid); ^{1}H NMR, δ 5.567 (dd, 5.7, 2.4 Hz, H-6), 5.385 (dt, 5.7, 2.7 Hz, H-7), 5.34 (m, H-8', H-9'), 4.713 (tt, 11.4, 4.5 Hz, H-3α), 2.493 (ddd, 14.5, 4.9, 2.3 Hz, H-4α), 2.28 (m, H-2'), 2.09

(m, H-12β), 0.953 (s, H-19), 0.941 (d, 6.6 Hz, H-21), 0.88 (br t, H-18'), 0.871 (d, 6.6 Hz, H-27), 0.867 (d, 6.6 Hz, H-26), 0.618 (s, H-18).

EXAMPLE 28

Methods for Sample Collection

[00157] SLOS blood samples were obtained with informed consent of the subjects or their parents, and the studies on human samples were performed with institutional review board approval. Samples of erythrocytes and plasma (ca. 20 ml each) were obtained from a 28-year-old SLOS subject before (A) and after (B) one month of a high cholesterol diet (target of 1050 mg cholesterol per day from meat, eggs, and dairy products). More limited analyses were carried out on a 0.5-ml whole blood sample from a 1-year old girl severely affected by SLOS (H) and 0.5-ml plasma samples from seven mildly affected SLOS subjects (I-O) and three normal subjects (S-U). Normal human serum (sample Z) was purchased from Sigma (St. Louis, MO). Other normal blood (samples V-Y) and plasma was obtained from donors. Blood samples were stored at -80°C before analysis.

EXAMPLE 29

Methods for Materials

[00158] The following C_{27} sterols were prepared as described previously (Wilson *et al.*, 1996) and showed purities of ≥98%: cholesta-5,7-dien-3β-ol acetate, cholesta-5,8-dien-3β-ol (8DHC), cholesta-5,8(14)-dien-3β-ol ($\Delta^{5,8(14)}$), 5α-cholesta-6,8-dien-3β-ol acetate ($\Delta^{6,8}$), 5α-cholesta-6,8(14)-dien-3β-ol acetate ($\Delta^{6,8(14)}$), 5α-cholesta-7,9(11)-dien-3β-ol acetate ($\Delta^{7,9(11)}$), 5α-cholesta-7,14-dien-3β-ol acetate ($\Delta^{7,14}$), 5α-cholesta-8,14-dien-3β-ol ($\Delta^{8,14}$), 5α-cholesta-8,24-dien-3β-ol acetate ($\Delta^{8,24}$), cholesta-5,7,9(11)-trien-3β-ol acetate ($\Delta^{5,7,9(11)}$), and 19-norcholesta-5,7,9-trien-3β-ol acetate. Except for 7DHC and 8DHC, these sterols were designated by their positions of unsaturation. Cholesterol was purified *via* the dibromide. In preferred embodiments, solvents and reagents were chromatographic or reagent grade; chloroform was hydrocarbon-stabilized from bottles less than 6 months old.

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[00159] The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference:

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PATENTS

U.S. Patent No. 5,629,210; issued May 13, 1997.

CLAIMS

We claim:

1. A method to screen a mammal for a genetic disorder of sterol metabolism comprising the steps of:

   a. collecting a test sample from said mammal to be tested;

   b. extracting sterols from said test sample;

   c. concentrating the sterol extract;

   d. forming a reaction mixture by mixing the concentrated sterol extract or a solution thereof with an acid reagent; and

   e. comparing a transient color of said reaction mixture with a transient color of a control mixture to determine the presence of said genetic disorder of sterol metabolism.

2. The method of claim 1, wherein said test sample is selected from the group consisting of a dried blood spot and a body fluid.

3. The method of claim 2, wherein said dried blood spot is on a Guthrie card, wherein said Guthrie card is treated with a chemical stabilizer.

4. The method of claim 1, wherein said genetic disorder is selected from the group consisting of Smith-Lemli-Opitz syndrome, Conradi-Hunermann syndrome, CHILD syndrome, desmosterolosis, and Greenberg dysplasia.

5. The method of claim 1, wherein said genetic disorder is Smith-Lemli-Opitz syndrome.

6. The method of claim 1, wherein said genetic disorder is Conradi-Hunermann syndrome.

7. The method of claim 1, wherein said extracting comprises the steps of:

   a. adding to said test sample about 16 volumes of methanol;

b. adding to said test sample about 30 to 50 volumes of chloroform; and

c. stirring for about 30 minutes or mixing on a vortex apparatus for about 1 minute.

8. The method of claim 1, wherein said acid reagent is Liebermann-Burchard reagent.

9. The method of claim 8, wherein said Liebermann-Burchard reagent is modified by diluting with chloroform.

10. The method of claim 1, further comprising mixing said concentrated sterol extract or a solution thereof with said acid reagent, wherein said concentrated sterol extract or solution thereof is at a first subambient temperature and said acid reagent is at a second subambient temperature.

11. The method of claim 1, wherein said mixing comprises a stopped-flow cell apparatus.

12. The method of claim 1, wherein said comparing comprises visualization of the transient color at about 5 seconds following the addition of said acid reagent.

13. The method of claim 1, wherein said comparing comprises spectrophotometric absorption detection of the transient color at about 2 minutes following the addition of said acid reagent.

14. The method of claim 13, wherein said absorption is detected at a wavelength of about 620 nanometers.

15. The method of claim 14, wherein said absorption is corrected for interference by subtracting an absorption at a second wavelength, or by subtracting an absorption at said 620 nanometers at a first reaction time from an absorption at said 620 nanometers at a second reaction time.

16. The method of claim 1, further comprising the step of drying said sterol extract with a solid drying agent.

17. The method of claim 16, wherein said solid drying agent is sodium sulfate.

18. The method of claim 1, further comprising the step of removing solid particles from the test sample, the sterol extract, or the reaction mixture.

19. A method to screen a human for Smith-Lemli-Opitz syndrome comprising the steps of:

a. collecting a test sample from said human;

b. extracting sterols from said test sample;

c. concentrating the sterol extract;

d. forming a reaction mixture by mixing said concentrated sterol extract with an acid reagent; and

e. comparing a transient color of said reaction mixture with a transient color of a control mixture to determine a positive test for Smith-Lemli-Opitz syndrome.

20. The method of claim 19, wherein said test sample is selected from the group consisting of a dried blood spot and a body fluid.

21. The method of claim 20, wherein said dried blood spot is on a Guthrie card, wherein said Guthrie card is treated with a chemical stabilizer.

22. The method of claim 19, wherein said extracting comprises the steps of:

a. adding to said test sample about 16 volumes of methanol;

b. adding to said test sample about 30 to 50 volumes of chloroform; and

c. stirring for about 30 minutes or mixing on a vortex apparatus for about 1 minute.

23. The method of claim 19, wherein said acid reagent is Liebermann-Burchard reagent.

24. The method of claim 23, wherein said Liebermann-Burchard reagent is modified by diluting with chloroform.

25. The method of claim 19, wherein said acid reagent comprises triflic acid or trifluormethanesulfonic acid at about 0.5% final volume.

26. The method of claim 19, wherein said acid reagent comprises sulfuric acid in the range of about 0.2% to about 8% final volume.

27. The method of claim 19, wherein said acid reagent comprises a superacid.

28. The method of claim 19, further comprising mixing said concentrated sterol extract or a solution thereof with said acid reagent, wherein said concentrated sterol extract or solution thereof is at a first subambient temperature and said acid reagent is at a second subambient temperature.

29. The method of claim 19, wherein said mixing comprises a stopped-flow cell apparatus.

30. The method of claim 19, wherein said comparing comprises visualization of a transient color at about 5 seconds following the addition of said acid reagent, wherein visualization of a pink color indicates a positive test for Smith-Lemli-Opitz syndrome.

31. The method of claim 19, wherein said transient color of the reaction mixture is determined by a spectrophotometer comprising a diode array detector.

32. The method of claim 19, wherein said comparing comprises spectrophotometric absorption detection of a transient color at about 5 seconds following the addition of said acid reagent.

33. The method of claim 19, wherein said comparing comprises spectrophotometric absorption detection of a transient color at about 5 seconds following the addition of said

acid reagent, wherein an absorbance at a first wavelength of about 510 nanometers corrected for interference by subtracting an absorbance at a second wavelength of about 560 nanometers is greater than about 1.150 AU indicates a positive test for Smith-Lemli-Opitz syndrome.

34. The method of claim 19, wherein said comparing comprises spectrophotometric absorption at a wavelength of about 620 nm, wherein said absorption is corrected for interferences by subtracting an absorption at a different wavelength, or by subtracting an absorption at said 620 nanometers at a different reaction time.

35. The method of claim 34, wherein said spectrophotometric absorption at about 620 nanometers is determined at about 2 minutes compared to spectrophotometric absorption at about 30 minutes following the addition of said acid reagent, wherein the ratio of the first absorbance and the second absorbance is greater than about 0.1 indicates a positive test for Smith-Lemli-Opitz syndrome.

36. The method of claim 19, further comprising the step of drying said sterol extract with a solid drying agent.

37. The method of claim 36, wherein said solid drying agent is sodium sulfate.

38. The method of claim 37, further comprising the step of removing solid particles from the test sample, the sterol extract, or the reaction mixture.

39. A method to diagnose Smith-Lemli-Opitz syndrome comprising the steps of:

a. collecting a test sample from said human;

b. extracting sterols from said test sample;

c. concentrating the sterol extract;

d. forming a solution by dissolving said concentrated sterol extract in a deuterated organic solvent; and

e. analyzing said solution by nuclear magnetic resonance, wherein the presence of a singlet at about 0.620 ppm indicates the presence of 7-dehydrocholesterol, wherein the presence of 7-dehydrocholesterol diagnoses said Smith-Lemli-Opitz syndrome.

40. The method of claim 39, wherein said analysis comprises a nuclear magnetic resonance spectrometer of at least about 300 MHz.

41. A method to diagnose Smith-Lemli-Opitz syndrome comprising the steps of:

a. collecting a test sample from said human;

b. extracting sterols from said test sample;

c. concentrating the sterol extract;

d. forming a solution by dissolving said concentrated sterol extract in a deuterated organic solvent; and

e. analyzing said reaction mixture by nuclear magnetic resonance, wherein the presence of a singlet at about 0.652 ppm indicates the presence of 8-dehydrocholesterol, wherein the presence of 8-dehydrocholesterol diagnoses said Smith-Lemli-Opitz syndrome.

42. The method of claim 41, wherein said analysis comprises a nuclear magnetic resonance spectrometer of at least 300 MHz.

43. A method to diagnose Conradi-Hunermann syndrome comprising the steps of:

a. collecting a test sample from said human;

b. extracting sterols from said test sample;

c. concentrating the sterol extract;

d. forming a solution by dissolving said concentrated sterol extract in a deuterated organic solvent; and

e. analyzing said solution by nuclear magnetic resonance, wherein the presence of a singlet at about 0.652 ppm indicates the presence of 8-dehydrocholesterol, wherein the presence of 8-dehydrocholesterol diagnoses said Conradi-Hunermann syndrome.

44. A method to treat a Guthrie card comprising impregnating said Guthrie card with a chemical stabilizer.

45. The method of claim 44, wherein said Guthrie card comprises a dried blood spot.

46. The method of claim 44, wherein said chemical stabilizer is selected from a group consisting of butylated hydroxytoluene, ascorbic acid, alpha-tocopherol, beta-carotene, mannitol, tert-butyl phenyl nitrone and N-acetyl cysteine.

47. The method of claim 44, wherein said chemical stabilizer is butylated hydroxytoluene.

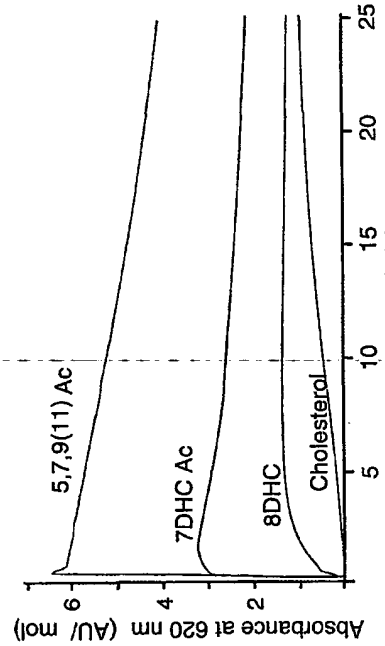
5,7,9(11) Ac
7DHC Ac
8DHC
Cholesterol
Absorbance at 620 nm (AU/ mol)
Time (min)

FIG. 1A

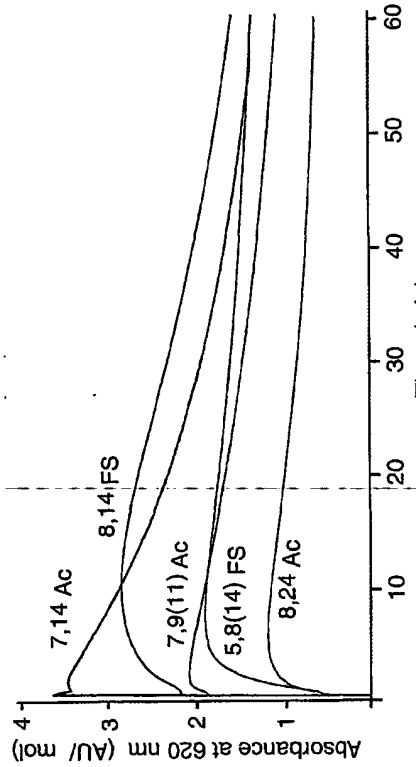
7,14 Ac
8,14 FS
7,9(11) Ac
5,8(14) FS
8,24 Ac
Absorbance at 620 nm (AU/ mol)
Time (min)

FIG. 1B

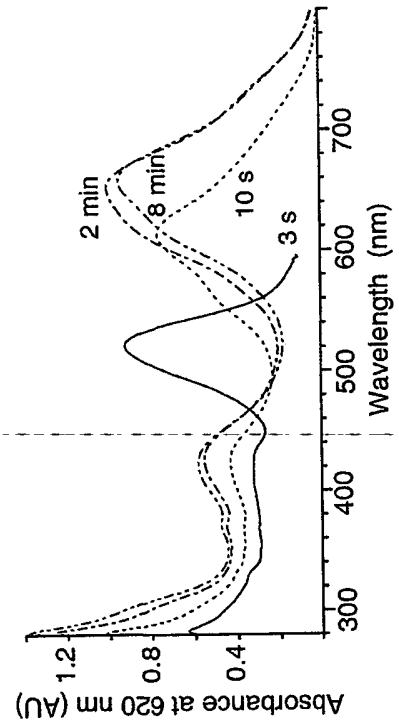
Absorbance at 620 nm (AU)
1.2
0.8
0.4
300
400
500
600
700
Wavelength (nm)
2 min
8 min
10 s
3 s

FIG. 2A

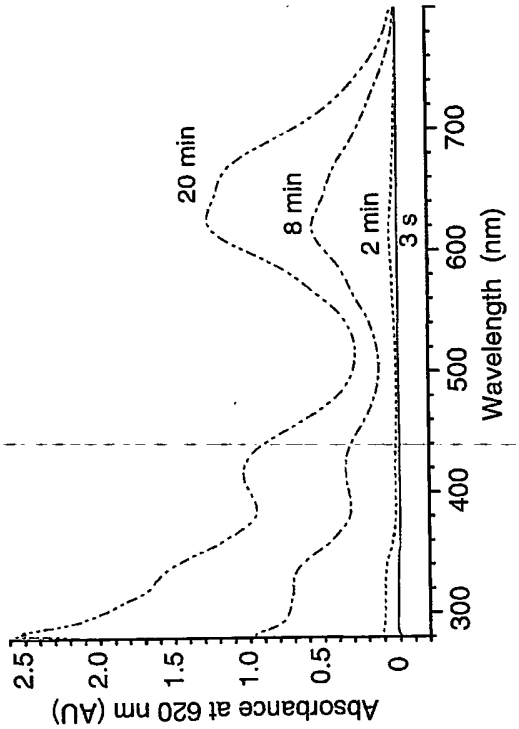
Absorbance at 620 nm (AU)
2.5
2.0
1.5
1.0
0.5
0
300
400
500
600
700
Wavelength (nm)
20 min
8 min
2 min
3 s

FIG. 2B

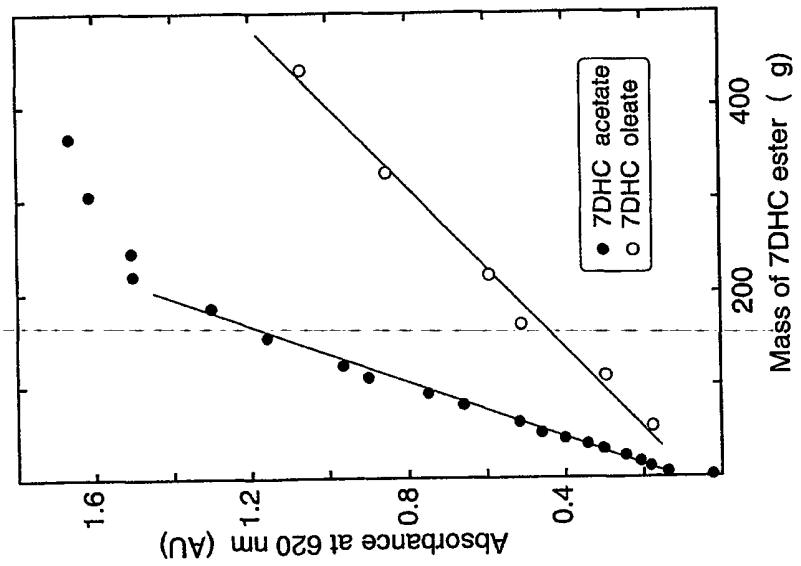
Absorbance at 620 nm (AU)
1.6
1.2
0.8
0.4
200
400
Mass of 7DHC ester (g)
7DHC acetate
7DHC oleate

FIG. 3

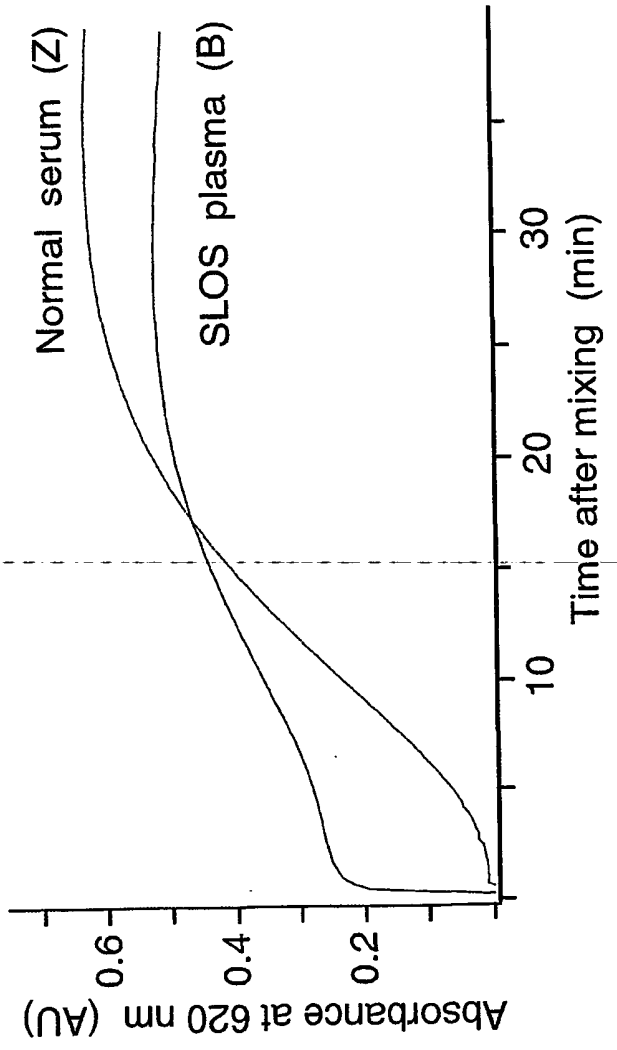
Normal serum (Z)
SLOS plasma (B)
Absorbance at 620 nm (AU)
0.2
0.4
0.6
10
20
30
Time after mixing (min)

FIG. 4

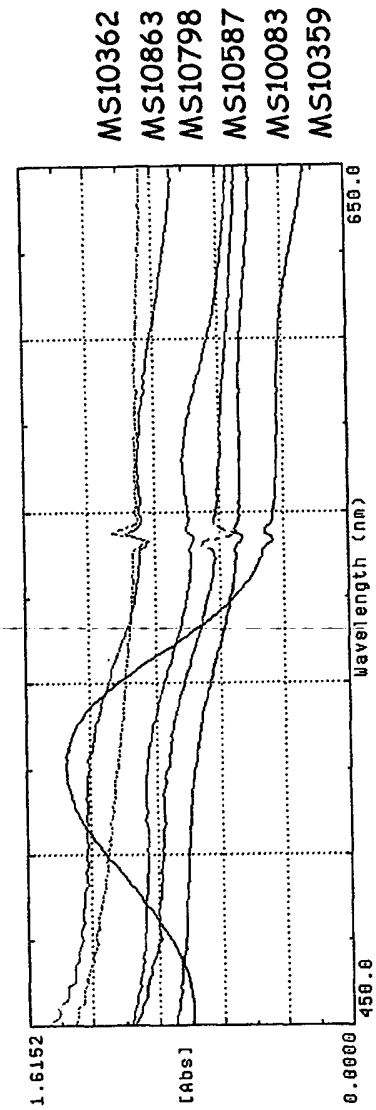
MS10362
MS10863
MS10798
MS10587
MS10083
MS10359
650.0
Wavelength (nm)
450.0
1.6152
[Abs]
0.0000

FIG. 5A

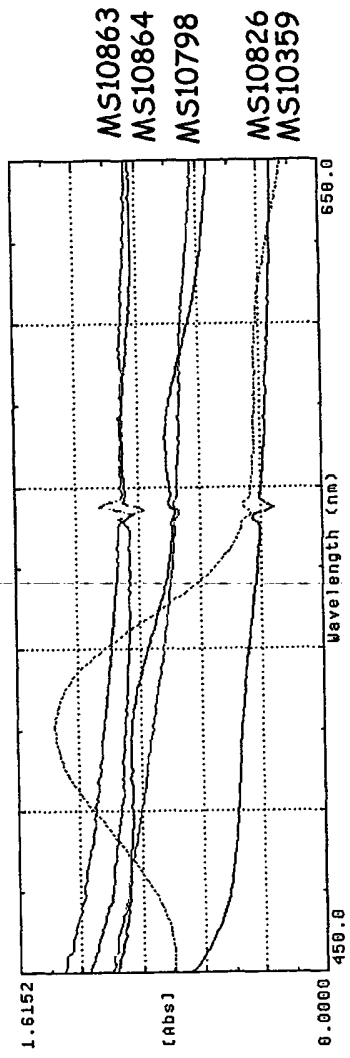
MS10863
MS10864
MS10798
MS10826
MS10359
650.0
Wavelength (nm)
450.0
1.6152
[Abs]
0.0000

FIG. 5B

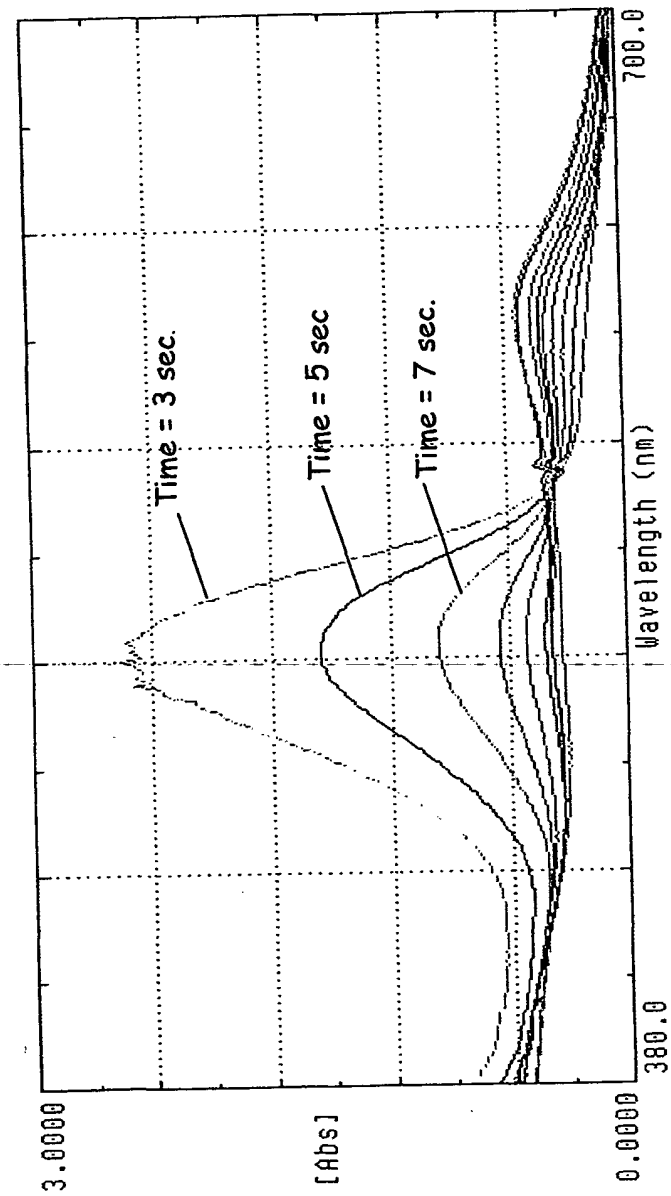
Time = 3 sec.
Time = 5 sec
Time = 7 sec.
3.0000
[Abs]
0.0000
380.0
700.0
Wavelength (nm)

FIG. 6

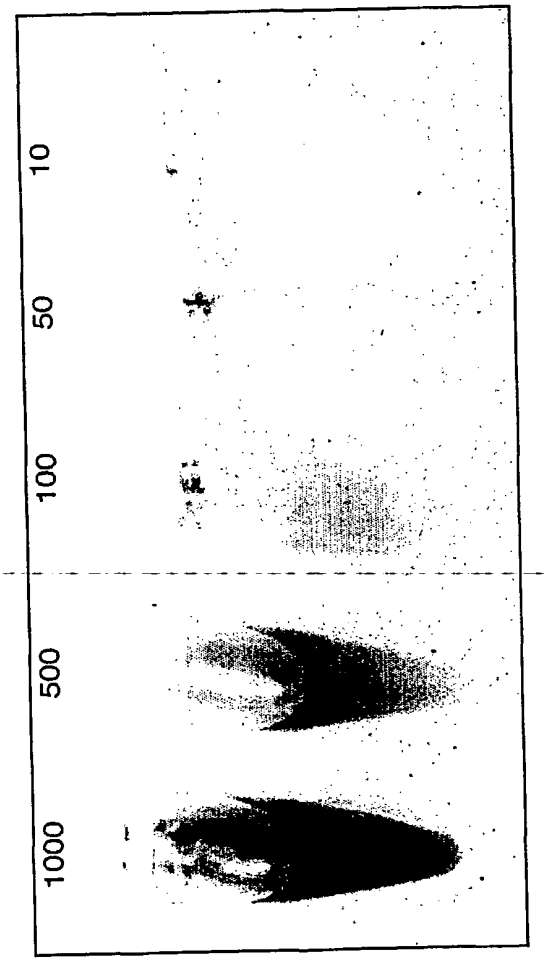

FIG. 7

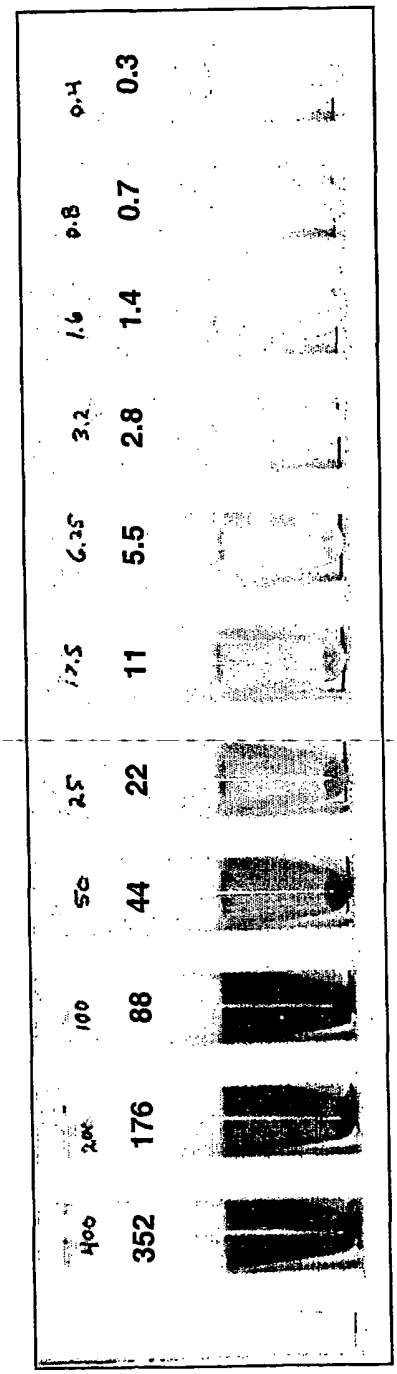
400
200
100
50
25
12.5
6.25
3.2
1.6
0.8
0.4
352
176
88
44
22
11
5.5
2.8
1.4
0.7
0.3

FIG. 8

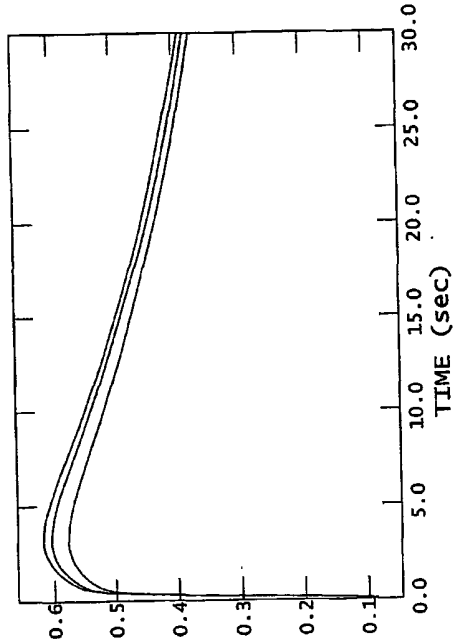
0.0
5.0
10.0
15.0
20.0
25.0
30.0
TIME (sec)
0.6
0.5
0.4
0.3
0.2
0.1

FIG. 9B

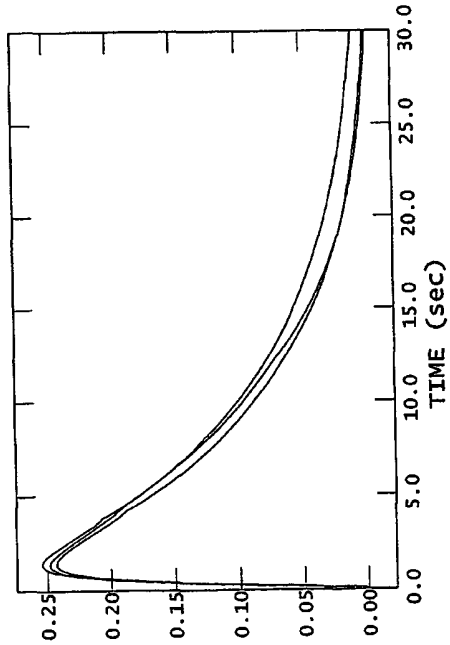
0.0
5.0
10.0
15.0
20.0
25.0
30.0
TIME (sec)
0.25
0.20
0.15
0.10
0.05
0.00

FIG. 9A

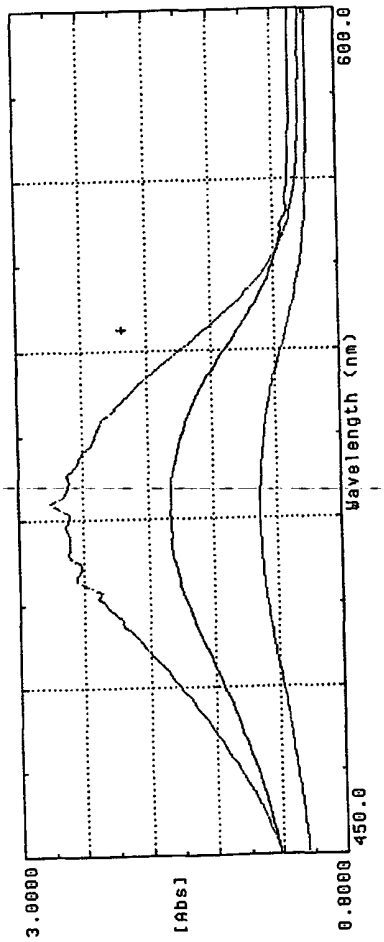

FIG. 10A

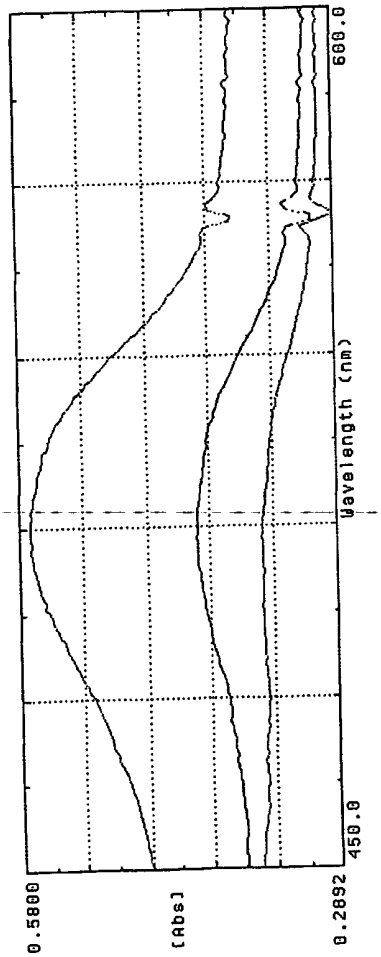

FIG. 10B

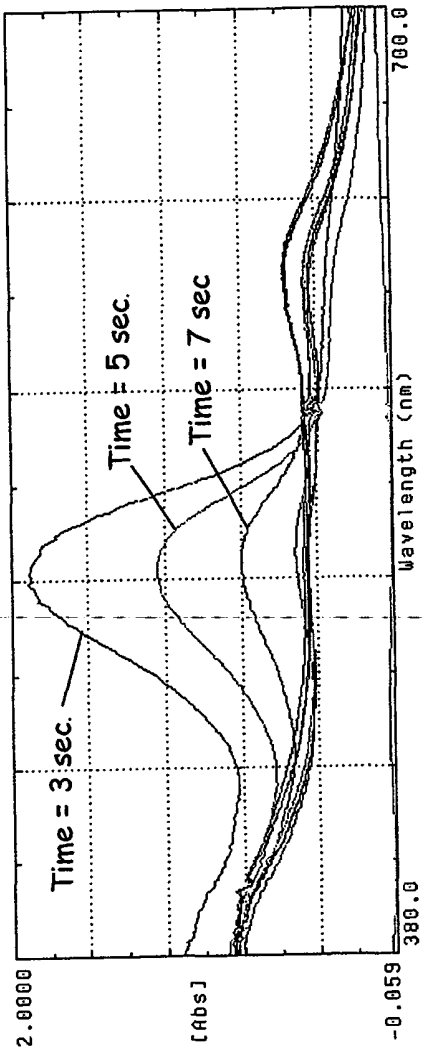

FIG. 11A

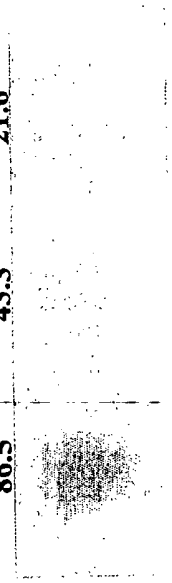

FIG. 11B

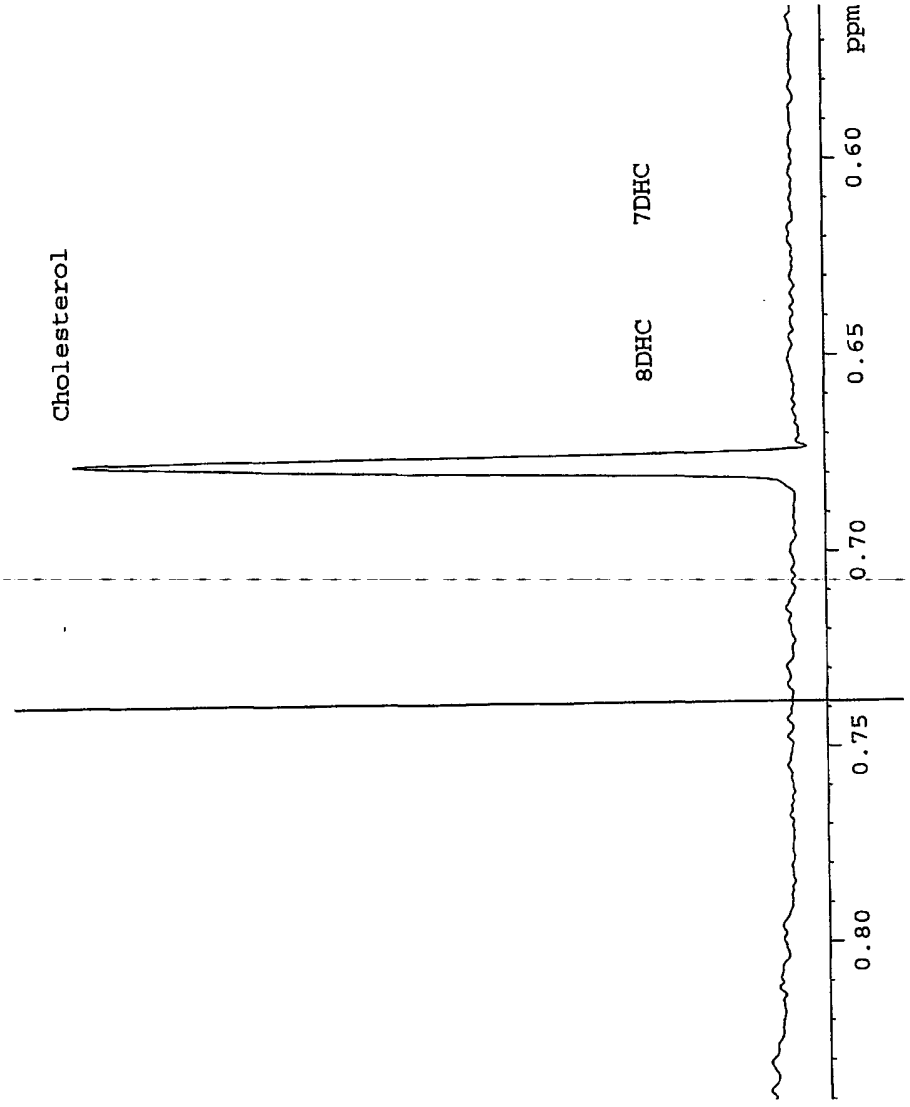

FIG. 12A

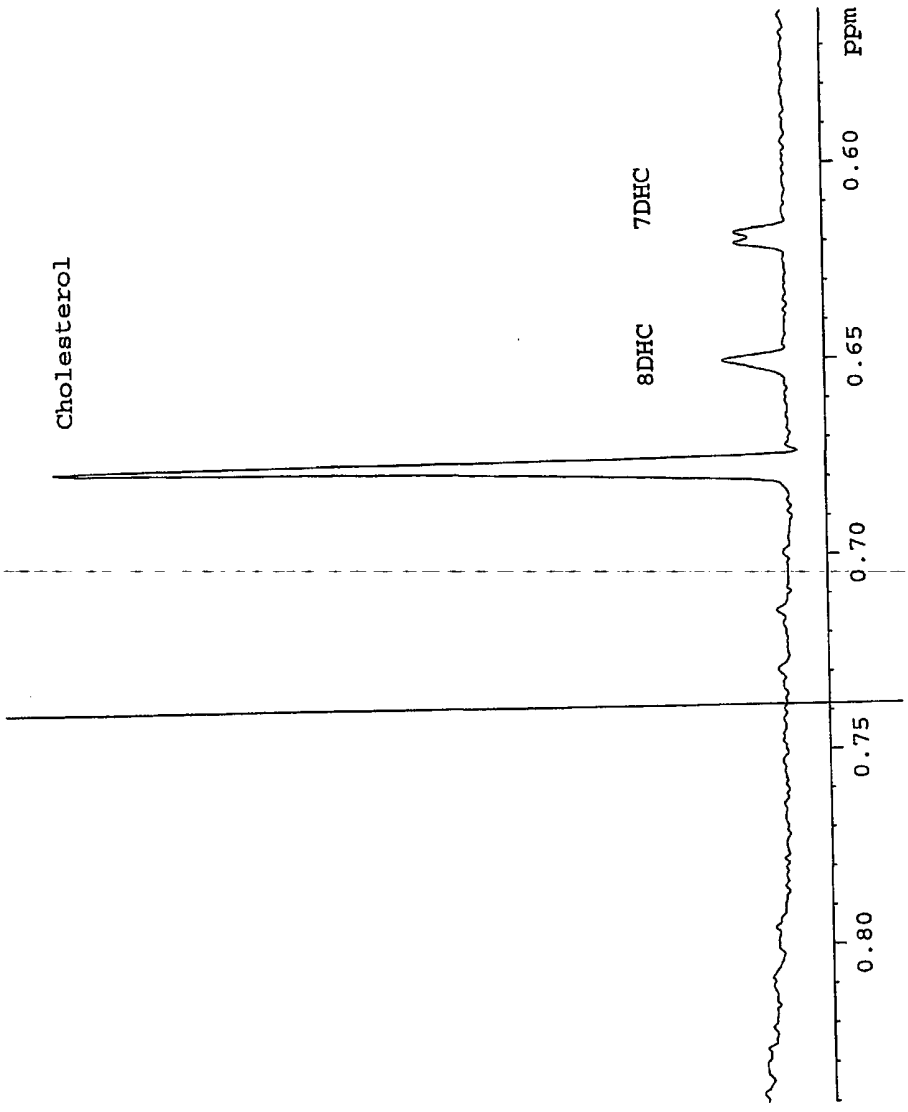

FIG. 12B

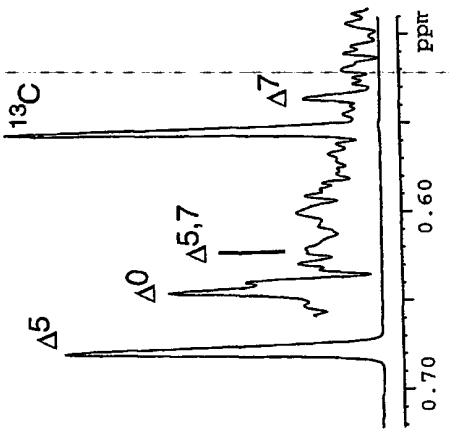

FIG. 13A

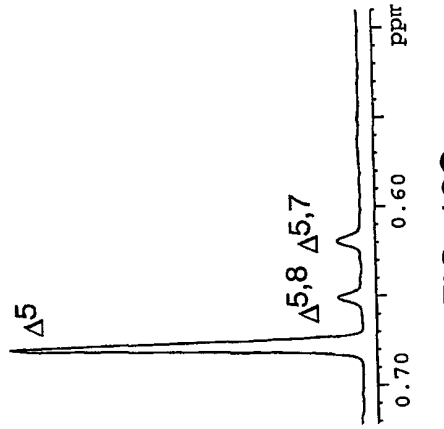

FIG. 13C

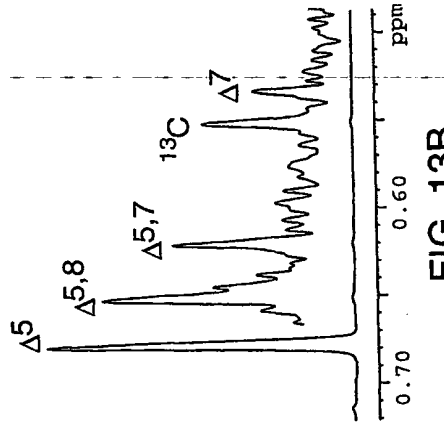

FIG. 13B

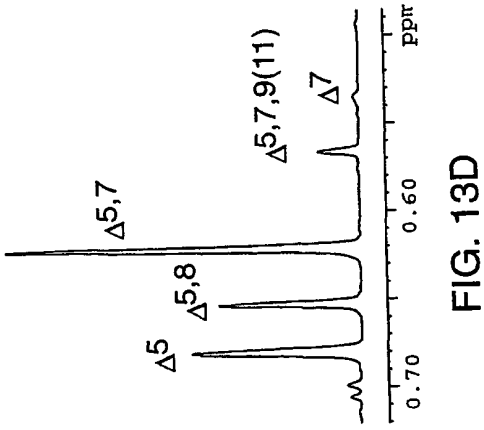

FIG. 13D

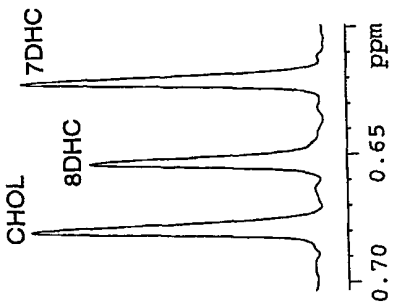

FIG. 14A

FIG. 14B

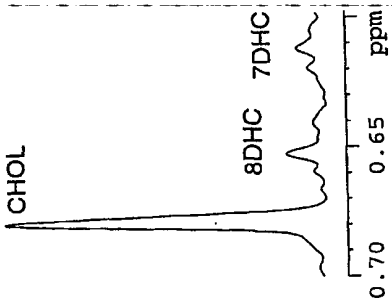

FIG. 14C

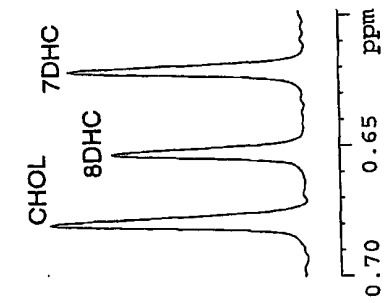

FIG. 14D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/17175

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : G 01 N 33/92
US CL : 436/815, 811, 164, 71
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 436/815, 811, 164, 71

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,629,210 A (HERCULES et al) 13 May 1997 (13.05.1997), abstract.	1-5 and 7-42

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 July 2001 (28.07.2001)	28 AUG 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Jill Warden Telephone No. 703-306-0661

Form PCT/ISA/210 (second sheet) (July 1998)